REMARKS

Applicants gratefully acknowledge the courtesy shown by the Examiner during the interview on April 25, 2000 with Dr. Daniel Meruelo, Paul Fehlner, and the undersigned, during which all of the rejections were discussed.

Reconsideration of this application is respectively requested.

In the Office Action mailed December 22, 2000, claims 1-10, 18-23 and 27-31 were rejected under 35 U.S.C. §§112 first paragraph and 103(a). Claims 1, 8 and 18 been amended. Support for this amendment of claims 1 and 18 can be found in numerous places in the specification as filed. For example, on page 25, line 24 it is stated "the new tropism of the recombinant virus . . .", on page 21, lines 14-17 is it stated "In this example we describe the construction of a recombinant Sindbis virus vector displaying protein A-envelope chimeric proteins to redirect the viral tropism". See also page 8, lines 24-27: ". . . these chimeric proteins enable one to use an antibody to target the viral particle to a desired cell to which the antibody binds and not to a cell to which the antibody does not bind." Also, page 9, lines 21-22 (description of Figure 3), which describes a " . . . schematic strategy for retargeting an Sindbis virus vector." See also page 4, lines 2-4: " . . . direct modification of the envelope protein of murine leukemia virus (MLV) have been shown to redirect the viral tropism." At page 6, lines 19-23: "It is desirable to alter the tropism of the Sindbis virus vectors to permit gene delivery specifically to certain target cell types. This will require both the ablation of endogenous viral tropism and the introduction of novel tropism". Claim 8 has been amended to correct its dependency (claim 8 was dependent from cancelled claim 7). Thus, no new matter has been added by this amendment. Claims 2-7 have been cancelled. Claims 1, 8-10, 18-23 and 27-31 are

pending in this application and are at issue.

The issues raised by the Examiner in the Office Action are as summarized and addressed below.

Claims 1-10, 18-23 and 27-31 were rejected under 35 U.S.C. § 112 first paragraph because the Examiner contended that the specification, while being enabling for the insertion of the particular "ZZ" IgG binding domain of Protein A into a viral vector, did not reasonably provide enablement for the use of any other IgG binding domain of Protein A. The Examiner contended that the specification did not disclose that any other IgG binding domain, or portion of any other IgG binding domain of Protein A, would be usable in the invention. This rejection is respectfully traversed and reconsideration is respectfully requested.

As discussed during the interview, there is no reason to doubt that those of ordinary skill in the art would be able to use the disclosure of the instant application with respect to incorporating any IgG binding domain of protein A into the Sindbis viral vectors disclosed and claimed herein should any other IgG binding domain of Protein A be substituted for the Z domain.

First, Staphylococcal Protein A (SpA), the cell wall bound type-I Fc receptor, exhibits tight binding to many IgG, IgA and IgM molecules at site(s) different from the antigen-combining site. As disclosed on page 15, lines 31 - 35 of the application, the extracellular part of SpA contains a tandem repeat of five highly homologous IgG-binding domains designated (from the N terminus) E, D, A, B and C, each of which includes about 58 amino acid residues. The Z domain is an engineered analog of the B domain originally developed as an affinity-purification handle for fusion protein production. Domains E, D, A, B, C and Z are all highly homologous

and bind the Fc domain of human IgG antibodies. The Z domain is well known to those of ordinary skill in the art and is commercially available on several plasmids, but otherwise is functionally the same as E, D, A, B and C. Thus, there is every reason to believe that any IgG binding domain derived from Protein A would be able to function in the same manner as the synthetic Z binding domain disclosed in the specification.

The complete sequence of the Staphylococcus protein A gene has been known since 1984 (*see* Uhlen *et al.*, appended hereto as Exhibit 1). Each of the five homologous domains is only 58 amino acids in length. Therefore, those of ordinary skill in the art would be able to obtain the DNA encoding each domain (either from the bacteria - it is available from the American Type Culture Collection, ATCC or synthetically synthesized), insert it into a viral vector as disclosed herein on Page 16, line 20-36 and assay for it as disclosed on p. 17, lines 20-31.

A second observation, published by the Applicant's laboratory, makes it clear that the system described and claimed herein is robust enough to accept a much broader range of modifications than would be contemplated by the simple replacement of a Z domain by any of the highly homologous domains of Protein A. In the Sawai and Meruelo article (appended hereto as Exhibit 2) a Sindbis virus vector was produced that targeted human choriocarcinoma cells via ligand-receptor interaction. In this case a much larger and entirely different molecular construct than ZZ was inserted within the Sindbis protein E2. The same Sindbis virus envelope gene as used in the instant specification [E2] was modified by insertion of the a and b-hCG genes (the a-linker-b-hCG construct utilized to modify the Sindbis E2 molecule is larger and far more complex than the ZZ domains of Protein A). Sawai and Meruelo demonstrated that a hCG-envelope chimeric virus could

indeed be produced and that this vector infected and transferred a reporter gene to choriocarcinoma cells as well as other cells bearing LH/CG receptors, but not to cells lacking these receptors. This publication clearly shows that the viral vectors claimed herein can accommodate domains or genes other than "ZZ" IgG binding domains and that, in fact, complex genes of substantial size are also well tolerated.

In the Advisory Action mailed July 29, 2001, the Examiner admitted that the above arguments were valid but the claims were drawn to all viruses. In fact, the Examiner withdrew his rejection of claims 7-10. In order to expedite prosecution and allowance of the claims pending in this case, Applicants have limited the claims to Sindbis virus. Claims 2-7, directed to a different embodiment of the present invention, have been cancelled, without prejudice or disclaimer and the dependency of claim 8 has been changed. Applicants reserve the right to pursue broader claims in a duly filed continuation application.

Therefore, Applicants respectfully request that the rejection of claims 1-10, 18-23 and 27-31 under 35 U.S.C. §112 first paragraph be withdrawn.

Claims 1-10, 18-23, and 27-31 were also rejected under 35 U.S.C. 103 (a) as being unpatentable over Barber *et al.* (US Patent 5,591,624) and Wickham *et al.* (US Patent 5,846,782), in view of Nilsson *et al.* 1987 Protein Eng. 1:107-113) . The Examiner contends that Applicant's arguments were against the references individually and that one cannot show nonobviousness by attacking references individually where the rejections are based on combinations of references. This rejection is respectfully traversed and reconsideration is respectfully requested.

As discussed during the interview, the present invention achieves specific targeting without cross-targeting due to the viruses' natural tropism, and yields viral vector that is competent to infect and transduce the target cells with high efficiency. It will be shown below that the references cited by the Examiner, either alone or in combination, do not provide one of ordinary skill a reasonable expectation of success in producing a viral vector for transducing a cell with high efficiency and which alters the natural viral tropism, as claimed herein. In addition, it will be shown that what is taught in Barber *et al.* (US Patent 5,591,624) and is being considered relevant to the Applicant's invention, fails to achieve the advantages of the claimed invention. What is taught by Wickham *et al.* (US Patent 5,846,782) also falls short of the claimed invention.

Neither Barber *et al.* nor Wickham *et al.* provided methodology to guide those of ordinary skill in the art to Applicant's invention as claimed herein. Despite many efforts, prior to the Applicant's invention no one had taught how to generate a highly efficient and versatile virus vector capable of targeting specific cells by a receptor-ligand interaction which alters natural viral tropism.

A. ***Barber et al.***

What Barber *et al.* teaches (Column 5, lines 6-40):

> "A technique suitable for producing recombinant retroviruses which can be targeted for preselected cell lines utilizes recombinant retroviruses having one or more of the following:
>
> an env gene comprised of a cytoplasmic segment of a first retroviral phenotype, and an extracellular binding segment exogenous to the first retroviral phenotype (the binding segment being from a second viral phenotype or from another protein with desired binding properties which is selected to be expressed as peptide which will bind to the desired target);

another viral envelope protein;

another ligand molecule in place of the normal envelope protein; or another ligand molecule along with an envelope protein that does not lead to infection of the target cell type.

Preferably, in the technique briefly described above, an env gene comprised of a cytoplasmic segment of a retroviral phenotype is combined with an exogenous gene encoding a protein having a receptor-binding domain to improve the ability of the recombinant retrovirus to bind specifically to a targeted cell type, e.g., a tumor cell.

In this regard, it may be preferable to utilize a receptor-binding domain which binds to receptors expressed at high levels on the surface of the target cell (e.g., growth factor receptors in tumor cells) or alternatively, a receptor-binding domain binding to receptors expressed at a relatively higher level in one tissue cell type (e.g., epithelial cells, ductal epithelial cells, etc. in breast cancer).

One potential advantage to targeting with hybrid enveloped with specificity for growth factor or activation receptors (like EGF or CD3 receptors) is that binding of the vector itself may then lead to cell cycling, which is necessary for viral integration and expression. Within this technique, it may be possible to improve and genetically alter recombinant retroviruses with specificity for a given tumor by repeated passage of a replication recombinant retrovirus in tumor cells; or by linking the vector construct to a drug resistance gene and selecting for drug resistance."

However, the specification provided by Barber *et al.* in fact does not teach how to generate chimeric vectors capable of targeted retroviral-mediated gene transfer

Barber et al.'s attempts to produce chimeric vectors capable of targeted retroviral-mediated gene transfer have consistently failed because they have been performed with insufficient knowledge. As explained by Zhao *et al.,* (Proc. Nat. Acad. Sci. USA 46:4005-4010, 1999) (a highlighted copy is appended hereto as Exhibit 3), Barber *et al.* fails because it merely focuses on vectors that will bind to molecules on the target cells, but does not consider other

requirements that such constructs must meet. Quoting from Zhao *et al.* On Page 4005 , 1st paragraph (emphasis added).

> "Several attempts have been made either to substitute or to insert a ligand (either peptide or a single-chain antibody) into the envelope protein of a retroviral vector so that the vector could then bind to a specific receptor on a designated cell type. In initial studies, antibodies were used to bridge the vector and the host cells. Because of the low efficiency, more recent studies have engineered the envelope protein in an attempt to change the tropism of the retroviral vector. A ligand to the erythropoietin receptor or to the heregulin receptor has been used to replace the binding domain of the murine leukemia virus (MuLV) ecotropic envelope protein to achieve transduction of target cells. Insertion of a single-chain antibody (ScFV) or a ligand into the N-terminal region of the envelope protein also has been used to target cell-surface molecules. In addition to the ecotropic Moloney murine leukemia virus (Mo-MuLV), the envelope protein of spleen necrosis virus has been used as a model system. However, although some of these studies report individual clones that reach a titer as high as 10^4 on target cells, it has not been possible to reliably generate vector preparation carrying chimeric envelope proteins that are able to produce titers higher than a few hundred on target cells. A number of laboratories have tested alternative insertion and replacement constructs with different single-chain antibodies and ligands. A significant titer on target cell has not been consistently achieved despite the ability of these chimera to bind to the target cells."

In considering the abundant number of failures in the literature, Zhao *et al.* explain (on Page 4005, 1st paragraph on right side):

> ..."it is thought that, after binding to receptor, Mo-MuLV envelope protein undergoes a conformational change that leads to fusion and core entry. Our data suggest that it is this conformational change that cannot occur in the chimeric envelope protein."

Failing to consider, demonstrate and/or teach how to achieve the required conformational change, is a flaw in the disclosure of Barber *et al.*, which therefore fails to provide one of ordinary skill a reasonable expectation of success. Hence the widespread failure of attempts to

achieve what Barber *et al.* disclosed. Zhao *et al.* makes these points quite clearly it states (on Page 4010, third full paragraph):

> "The conclusion from these data is that the binding of the chimeric envelope protein with receptor on the target cell does not, in itself, lead to successful fusion of the viral and cellular membranes, a prerequisite for the transfer of the viral core into the target cell...Before efficient targeted transduction by using retroviral vectors can be achieved, it will probably be necessary to develop a much better understanding of the structure/function of the wild-type envelope protein itself. Recent x-ray crystallographic studies of the ecotropic envelope protein, although providing only structural information of parts of the envelope protein are valuable contributions to this understanding."

These observations explain the widespread failure of attempts to achieve what Barber *et al.* taught.

How does the Applicant's invention overcome the limitations described above?

The mechanism by which Sindbis vectors enter cells is entirely different from that of retroviruses. This fact, in addition to the fact that the Sindbis envelope is functionally and structurally quite distinct from that of retroviruses, makes it possible to overcome the block to gene transfer that is seen with retroviruses.

Retrovirus binding at the cell membrane requires a conformational change in the env molecule that allows fusion between the viral and cell membranes. Extensive experimentation has established that the normal pathway of entry for alphaviruses is quite different and involves endocytosis in clathrin-coated vesicles followed by transfer to endosomes. There the low pH leads to the fusion domain in E1 being exposed and the virus envelope fusing with the endosomal membrane. Thus, Sindbis entry does not require fusion at the cell surface; mere binding to a cell receptor will lead to entry by receptor-mediated endocytosis.

The SU subunit (also known as gp70 or env), which is the one Barber *et al.* focused its attention on, is responsible for receptor binding by retroviruses. On the surface of the virion, MuLV envelope proteins appear to exist as trimeric complexes of SU-transmembrane (TM) heterodimers. The TM subunit anchors the dimer in the membrane and contains the so called "fusion peptide", similar to that found in influenza hemagglutinin. Upon binding of the retroviral env protein to its receptor, a conformational change occurs that allows the TM subunit to trigger fusion between the viral and cellular membranes, thus releasing the viral core into the cytoplasm. This interaction is so constrained that changes to the SU protein are not well tolerated, if at all. While Sindbis infection does require fusion with the endosomal membrane, E2, which is the Sindbis envelope molecule modified by Applicant, plays no role in fusion, which is solely driven by pH effects on E1. The Sindbis fusion element E1 is unchanged by the Applicant's modifications. The presumption is that the fusion domain of E1, which is very hydrophobic, inserts into the target endosomal membrane and induces fusion of the two bilayers. This approach, which had not been previously proposed is why, as taught by the Applicant, modifications to E2 that affect binding to cell surface ligands allow successful retargeting of Sindbis virus and vectors, have no impact on fusion and consequently do not diminish infectious titers of the vector. Moreover, insertion into the retroviral envelope protein as taught in this invention preserves its membrane fusion activity.

Barber *et al.* does not teach how to make a targetable retrovirus virus that can actually bind to and inject the targeted cells with adequate efficiency.

Barber *et al.* is, in fact, more incomplete than the above analysis indicates. It is uncertain as to whether the Barber *et al.* methods can even be relied on to generate a virus

capable of binding a targeted receptor by the use of "an env gene comprised of a cytoplasmic segment of a first retroviral phenotype, and an extracellular binding segment exogenous to the first retroviral phenotype (the binding segment being from a second viral phenotype or from another protein with desired binding properties which is selected to be expressed as peptide which will bind to the desired target)."

As discussed by Wu *et al., Virology 269:7-17, 2000,* on Page 7 in the Abstract (a highlighted copy is appended hereto as Exhibit 4)

> "Targeting of retroviral vectors to specific cells has been attempted through engineering on the surface (SU) protein of the murine leukemia viruses (MuLVs), but in many cases this has adversely affected protein function and targeted delivery has been difficult to achieve."

These authors undertook a comprehensive screen to identify sites in the receptor-binding domain (RBD) of the MoMuLV env protein that could accommodate the insertion of a targeting peptide. The choice of sites was based on considerations of the 3D structure of the highly homologous RBD of FrMuLV, which was not available at the time Barber *et al.* filed the application, together with data from previous attempts to engineer MoMuLV env. Despite these and other considerations, the majority of the peptide insertions made by Wu *et al.* resulted in chimeric env proteins that were not properly processed or able to be incorporated into viral particles. As they state on Page 13, "these results emphasize the difficulties inherent in engineering a complex oligomeric protein, even when structural information is available." The studies of Wu *et al.* were successful only in delineating portions of the env gene that may be amenable to substitutions allowing retargeted binding, but not infection. As the authors state on Page 14: "However, even if high-affinity binding could be obtained, it remains a distinct possibility that this will not lead

to the fusion of viral and cellular membranes." Indeed, some of these investigators had previously demonstrated that the block to transduction for chimeric env proteins is due to the inability of the chimeric receptor interaction to trigger the env protein to a fusogenic state.

Applicant does teach how to make a targetable retrovirus virus that can actually bind the targeted cells.

Given these facts, it is clear that Applicant's invention as claimed herein is not disclosed or suggested in Barber *et al.* Applicants have taught specifically how to routinely make a versatile, targetable retrovirus or Sindbis vector capable of binding to cells with high affinity. Applicant has identified the site of insertion which allows this to happen (i.e. for Sindbis virus on Page 20, lines 16 - 21 of the instant Application, and for retrovirus on Page 12, lines 5 - 7) and has demonstrated that such a chimeric retrovirus or Sindbis can interact with most antibodies, picking up the antibody's high affinity binding properties for the cell surface antigen in question. Such vectors, containing the protein A binding domain, allows use of diverse targeting antibodies. Hence, the invention represents a unique solution to difficult and unpredictable tasks and one that was neither achieved nor taught by Barber *et al.*

B. Wickham *et al.*

What Wickham *et al.* actually teach:

Wickham *et al.* stated (column 3, lines 46-61) that:

> "The present invention provides a chimeric adenoviral fiber protein which differs from the wild-type (i.e., native) fiber protein by the introduction of a nonnative amino acid sequence in a conformationally-restrained (i.e. constrained) manner. The introduction results in the insertion of, or creation of, a constrained peptide motif that confers upon the resultant chimeric adenovirus fiber protein an ability to direct entry of a vector comprising the chimeric protein that is more efficient than entry into cells of a vector that is identical except for comprising a wild-type adenovirus

fiber protein, and/or an ability to direct entry into cells that adenovirus comprising the wild-type fiber protein typically does not infect/transduce. The present invention also provides vectors that comprise the chimeric adenovirus fiber protein, and methods of constructing and using such vectors."

Points of the Applicant's invention not addressed by the Wickham *et al.* patent and what the later patent fails to teach.

There are several important points that distinguish the Wickham *et al.* patent from Applicant's claims. First the Wickham *et al.* patent concerns itself solely with modifications to the adenoviral fiber protein, which is not part of the Applicant's claims, which are directed to chimeric envelope proteins containing an IgG binding domain of protein A.

Second, even with regard to the adenoviral fiber protein modifications proposed for targeting, Wickham *et al.* fails to teach how to achieve targeting specificity while altering adenoviruses natural binding activity as claimed herein. In contrast, the Applicant's claims call for altering the natural binding activity of Sindbis virus to its high affinity laminin receptor.

The failure of Wickham and Barber to teach using chimeric envelope proteins containing the IgG binding domain of protein A is not cured by Nilsson *et al.* No combination of the references would lead one of ordinary skill in the art to the present invention as claimed herein. Applicants respectfully submit that the review article by Wickham (in Nature Biotechnology appended hereto as Exhibit 5) is a strong indication of the non-obviousness of the present invention as claimed herein.

In his review article, Wickham praises the system as disclosed and claimed herein. Wickham concludes, on Page 717, last paragraph:

"In the result of such studies will not only be useful for those working with individual vector targeting systems, but especially for

those interested in applying gene targeting technology to the treatment of human disease"

This unsolicited (by the instant inventors) testimony can be considered to be similar to a Declaration. However, a Declaration in a patent application is only seen by the Examiner and those who obtain the file wrapper whereas this article is in a high profile peer reviewed, journal.

Another important difference between the work of Wickham *et al.* and the Applicant's is the nature of the molecules reengineered. It is not merely that Wickham *et al.* concerns itself solely with modifications of the adenoviral fiber protein. The differences are more significant, as the Applicant's invention considers the retargeting of viral envelope proteins. A copy of the paper discussed by Wickham *et al.* (Ohno et al) is appended hereto as Exhibit 6, while Wickham *et al.* deals with adenovirus vectors, which are non-enveloped.

It follows from the distinctive properties and structural arrangement of the Sindbis E2 glycoprotein, the retrovirus env (gp70), and the adenovirus fiber protein that it would be virtually impossible to translate observations from experiments with the latter to the former two. For example, whereas it has been argued that targeting might require a deletion of the terminal knob of the capsid protein fiber of adenovirus, which is required to alter this binding to cell, a deletion of the terminal portion of Sindbis E2 protein would probably have deleterious effects of the assembly of the external icosahedral lattice, as well as of the internal nucleocapsid protein lattice, which depends on associations with E2 for its formation. Thus, what Wickham *et al.* teaches is not only incomplete in terms of adenovirus retargeting, but also has no bearing on Sindbis retargeting strategies.

Appended hereto as Exhibit 7 is a Declaration from Dr. Daniel Meruelo, co-inventor of the above-identified patent application. In his Declaration, Dr. Meruelo describes results of a flow cytometry assay using a Fluorescence Activated Cell Sorter (FACS) showing that tumor cells were specifically targeted *in vivo* using the Sindbis viral vectors as disclosed and claimed in the instant application (Meruelo Declaration paragraph 8).

In these experiments, human colon carcinoma cells were inoculated into nude mice which express on their cell surface a CEA antigen (Meruelo Declaration, paragraph 5). A Sindbis viral vector as disclosed and claimed herein (Specification page 21, line 34 and page 22, line 21), was complexed with anti-CEA antibodies and injected into the mice. As controls, the mice received either antibody or the viral vector alone. In this case, the vector also contained a β-galactosidase reporter gene. The tumor cells were then isolated and analyzed for the presence the CEA antigen and the β-galactosidase gene.

Tumors from mice receiving only antibody and only virus did not stain positively for β-galactosidase, showing that they were not successfully targeted. However, about 73% of tumor cells positive for CEA antigen from mice injected with the virus-antibody complex were positive for β-galactosidase (Meruelo Declaration, paragraph 9).

The data clearly show that introducing an IgG binding domain from a Protein A into a Sindbis viral envelope protein provided for highly selective targeting. Dr. Meruelo concludes that the invention as disclosed and claimed herein, improves efforts at targeting viral vectors *in vivo* and achieves a level of targeting selectivity and infection efficiency that had not been previously described.

Finally, the Examiner has mentioned on Page 5 of Office Action that the limitation "high efficiency" was a latent property in the prior art and an advantage which would flow naturally from following the suggestion of the prior art. However, Applicants respectfully submit that this is a design choice required for the successful use of the claimed viral vectors which the prior art viral vectors lacked.

Conclusion

In sum, both Wickham *et al.* and Barber *et al.* fail to teach how to properly engineer the claimed vector molecules in order to achieve highly specific and efficient targeted viral vectors while altering natural viral tropism. Further, no combination of the teachings of these patents can be construed that could have provided those of ordinary skill in the art with the required knowledge to generate the invention as claimed herein. The Barber *et al.* approach has been attempted repeatedly by various groups and it has been widely reported to fail at achieving gene transduction of any significance. As knowledge in this area has increased it has become apparent that these failures are due to the fact that the retroviral gp70 molecule is not amenable to changes, and that altering its structure, other than as taught herein, abolishes it ability upon binding to trigger the fusogenic activity of the TM protein. Without fusion infection cannot occur. Further, even synthesis of the protein and its ability to bind are severely affected by most changes.

The Wickham et al patent also omits crucial elements of successful construct of this invention, i.e., altering the natural binding activity of the virus. Its proposed modifications depend on the existence of the viral fiber in the retargeted virus, which in fact, as demonstrated by others, must be nullified or eliminated to remove the natural binding activity of adenovirus.

Further, modified adenoviral vectors such as those proposed by Wickham *et al.* have been deemed too unstable to achieve in vivo targeting activity. Extensions of proposed modifications by Wickham *et al.* of a capsid fiber protein of an non-enveloped virus, such as adenovirus, to an envelope virus like a retrovirus or Sindbis virus are also an overreach.

Neither patent provide the teachings or even suggest the basis for the Applicant's invention, which was first to teach how to generate a highly efficient and versatile Sindbis virus vector capable of targeting specific cells by receptor-ligand interactions while altering the normal vial tropism as claimed herein.

Based on the above amendments and remarks reconsideration of this application and issuance of a Notice of Allowance is respectfully requested.

Respectfully submitted,

Dated: June 22, 2001

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File No.:5986/0I124

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of:

Daniel MERUELO and Kouichi OHNO

Serial No.: 08/829,558 Group Art Unit: 1645

Filed: 3/28/97 Examiner: R. Zeman

For: VIRAL VECTORS HAVING CHIMERIC ENVELOPE PROTEINS CONTAINING THE IgG-BINDING DOMAIN OF PROTEIN A

MARKED-UP AMENDMENT UNDER 37 C.F.R. §1.121

Hon. Commissioner of Patents
and Trademarks
Box AF
Washington, D.C. 20231

1. (Twice Amended) A Sindbis viral vector for transducing a target cell with high efficiency which comprises a gene encoding a chimeric envelope protein containing an IgG-binding domain of protein A sufficient to bind an Fc domain of an antibody with strong affinity [in which] wherein the envelope protein is a [viral] Sindbis virus envelope protein and wherein

the envelope protein is operable to direct the assembly of the [fragment] protein into a viral particle[.];

and wherein said chimeric envelope protein alters natural viral tropism.

8. (Amended) The viral vector of claim [7] 1, wherein the portion of the IgG binding domain of Protein A is inserted into an E2 glycoprotein of the Sindbis virus envelope protein.

18. (Twice Amended) A chimeric Sindbis virus for transducing a target cell with high efficiency which comprises a gene of interest under the control of an appropriate viral sequence and a chimeric protein comprising a chimeric envelope protein containing an IgG-binding domain of protein A sufficient to bind an Fc domain of an antibody with strong affinity and wherein said chimeric envelope protein alters natural viral tropism.

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Exhibit 1

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 259, No. 3, Issue of February 10, pp. 1695-1702, 1984
Printed in U.S.A.
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Complete Sequence of the Staphylococcal Gene Encoding Protein A

A GENE EVOLVED THROUGH MULTIPLE DUPLICATIONS*

(Received for publication, August 4, 1983)

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The gene coding for protein A from *Staphylococcus aureus* has been isolated by molecular cloning, and a subclone containing an 1.8-kilobase insert was found to give a functional protein A in *Escherichia coli*. The complete nucleotide sequence of the insert, including the structural gene and the 5′ and 3′ flanking sequences, has been determined. Starting from a TTG initiator codon, an open reading frame comprising 1527 nucleotides gives a preprotein of 509 amino acids and a predicted M_r = 58,703. The structural gene is flanked on both sides by palindromic structures followed by a stretch of T residues, suggesting transcriptional termination signals. Thus, it appears that protein A is translated from a monocistronic mRNA.

The sequence reveals extensive internal homologies involving a 58-amino acid unit, responsible for IgG binding, repeated 5 times and an 8-amino acid unit, possibly responsible for binding to the cell wall of *S. aureus*, repeated 12 times. Comparisons between the repeated regions show a marked preference for silent mutations, indicating an evolutionary pressure to keep the amino acid sequence preserved. The structure of the gene also suggests how the gene has evolved.

Evolution by gene duplication is a well known phenomenon among eukaryotic genes. The globin clusters, the immunoglobulins, and the interferon genes probably all have ancestral genes which have been duplicated and then diverged into functionally distinct genes (1). Examples of internally, repetitive sequences have also been reported; rabbit skeletal tropomyosin contains a 7-residue amino acid periodicity throughout the molecule (2), and similar repeats have been reported for chicken fibronectin (3) and mammalian serum albumin (4). Among prokaryotes, most reports of duplicated genes have involved *in vitro* constructions (5), which seem to be stable in *Escherichia coli*, but dramatically unstable in *Bacillus subtilis* (6). However, the amino acid sequences of a few cell wall-bound proteins from Gram-positive bacteria have revealed remarkable periodicity, *i.e.* staphylococcal protein A (7, 8) and streptococcal M protein (9).

We have earlier reported on the molecular cloning of the gene for staphylococcal protein A in *E. coli* (10). This protein interacts with the F_c (constant part of immunoglobulins) domain of several immunoglobulins from many species including man and has therefore been used extensively for quantitative and qualitative immunological techniques (11). Amino acid sequence analysis of protein A revealed two functionally distinct regions of the molecule (7, 8). Both regions have remarkably repetitive structures.

The NH_2-terminal part contains four or five homologous IgG-binding units consisting of approximately 58 amino acids each. The COOH-terminal part which is thought to bind to the cell wall of *Staphylococcus aureus* consists of several repeats of an octapeptide (Glu-Asp-Gly-Asn-Lys-Pro-Gly-Lys) (8).

In a previous report (10), we determined the nucleotide sequence of the promoter region, as well as the region coding for the NH_2-terminal part of the protein. Here we report the complete nucleotide sequence of the protein A gene including the 5′ and 3′ flanking regions from the *S. aureus* strain 8325-4. The structural gene is 1,527 nucleotides long giving a preprotein consisting of 509 amino acids and a M_r = 58,703. The repetitive structure of the gene has been clarified which suggests how the gene has evolved.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Plasmids—*E. coli* strains HB101 (12) and GM161 (13) were used as bacterial hosts. The plasmid vectors were pBR322 (14), pTR262 (15), and pEMBL9 (16).

DNA Preparations—Plasmid DNA was prepared by the alkaline extraction method (17). Transformation of *E. coli* was made as described by Morrison (18). Restriction endonucleases, T4 DNA ligase (New England Biolabs), alkaline phosphatase, and T4 polynucleotide kinase (Boehringer-Mannheim) were used according to the suppliers' recommendations.

Isolation of the 2.15-kilobase DNA fragment containing the entire protein A gene was made by digesting the plasmid pSPA3 (10) with *Eco*RV. The digested material was electrophoresed on a 5% polyacrylamide gel, and the 2.15-kilobase fragment was eluted electrophoretically. The isolated fragment was passed over an anion exchange column, eluted, and precipitated with ethanol. The precipitated material was washed in 80% ethanol, dried, resuspended in water, and used for DNA sequence analyses.

DNA Sequencing Determinations—DNA fragments were sequenced by the method of Maxam and Gilbert (19) or Sanger *et al.* (20). The samples were analyzed on 6, 8, and 20% denaturing polyacrylamide gels using the thermostatic LKB Macrophor system.

Computer Analysis—All the sequencing analyses were performed on a Hewlett-Packard desktop computer (HP-85) equipped with a HP7225A plotter. The software was constructed by M. Uhlén.

RESULTS AND DISCUSSION

DNA Sequence—We have earlier reported that the protein A gene from *S. aureus* strain 8325-4 is located on a 1.8-kilobase insert of staphylococcal DNA cloned in the plasmid

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¶ Supported by grants from the Swedish National Board for Technical Development.

‖ Present address, European Molecular Biology Laboratory, Heidelberg, Federal Republic of Germany.

** Supported by grants from the Swedish Medical Research Council and Pharmacia Fine Chemicals, Uppsala.

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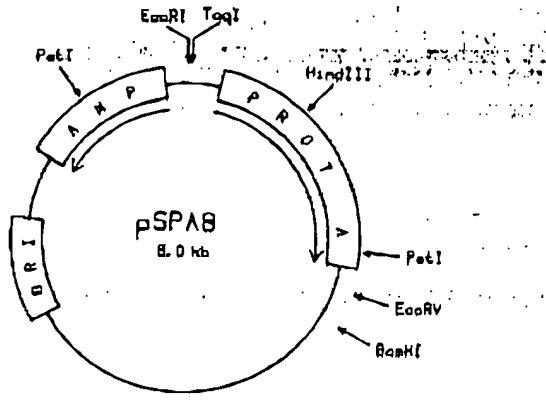

FIG. 1. **Structure of plasmid pSPA8 with relevant restriction sites.** The protein A gene is contained in a 1.8 kilobase *TaqI-EcoRV* insert in the plasmid pBR322. *Boxes* show the positions of the replication origin (*ORI*) and the genes coding for protein A (*PROT A*) and β-lactamase (*AMP*).

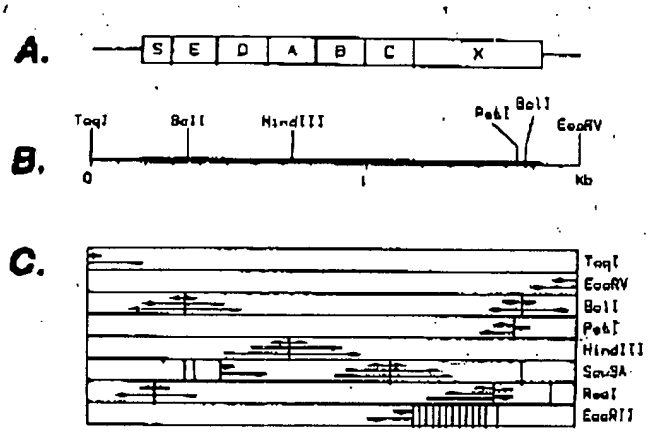

FIG. 2. **Restriction map and sequencing strategy of the insert.** *A*, schematic drawing of the gene coding for protein A with its different regions. *S* is a signal sequence, *A–D* are IgG-binding regions, *E* is a region homologous to *A–D*, and *X* is the COOH-terminal part of protein A which lacks IgG-binding activity. *B*, partial restriction map of the corresponding DNA sequence. *C*, sequencing strategy of the 1.8-kilobase insert.

pBR322 (21). The plasmid was designated pSPA8 and is shown schematically in Fig. 1. Expression of the gene was demonstrated in *E. coli*. The sequence of the promoter region and the 5′ end of the structural gene has been reported (10) as well as the sequence of the repetitive region X which probably is responsible for the cell wall binding of the protein in *S. aureus*.[1]

Using the strategy outlined in Fig. 2*C*, the entire insert was sequenced according to the method of Maxam and Gilbert (19). It was not possible to obtain sequence on both strands in all parts of the gene, and therefore additional sequencing using the enzymatic method (20, 16) was performed in order to confirm the sequence in these parts. As no palindromic sequence indicating transcription termination was found in the 3′ end of the gene, the sequence a few hundred nucleotides downstream from the *Eco*RV site on the original plasmid pSPA1 (10) was determined using both methods (19, 20). The complete nucleotide sequence of the protein A gene is shown in Fig. 3. Note that the previously published sequence of Löfdahl *et al.* (10) lacks one of the three thymidines at position 183–185.

[1] Guss, B., Uhlén, M., Nilsson, B., Lindberg, M., Sjöquist, J., and Sjödahl, J. (1984) *Eur. J. Biochem.*, in press.

Starting from a TTG codon at nucleotide 184, there is an open reading frame of 1,527 nucleotides terminating in a TAG stop codon at nucleotide 1,711. The preprotein, including the putative signal peptide, consists of 509 amino acids giving a M_r = 58,703. Although we have not shown that the codon at nucleotide 184 is the translational start, there are several reasons to postulate this. First, TTG is a common start codon in Gram-positive bacteria (21), unlike *E. coli* in which it is very rare (22). Second, this start codon gives a putative signal peptide with a reasonable size (36 amino acids) and structure (a few basic residues followed by a stretch of 23 hydrophobic residues). Third, this codon is preceded by a possible Shine-Dalgarno sequence (23) that has many features in common with other Gram-positive ribosomal binding sequences (24). 8 out of 11 nucleotides are complementary to the 3′ end of *B. subtilis* 16 S rRNA, similar to other Gram-positive genes (25). In addition, the space between the last G in this sequence and the start codon is seven nucleotides, also similar to other Gram-positive genes (24, 25).

Two upstream overlapping promoter sequences similar to the consensus sequences (TTGACA and TATAAT) of prokaryotes (26) have been indicated in Fig. 3, although the first −35 sequence shows relatively poor complementarity (only three out of six) with TTGACA. The gene is both preceded and followed by palindromic sequences indicating transcription terminations. These are indicated in Fig. 3, and the possible mRNA hairpin structures that can be formed are schematically drawn in Fig. 4. Both palindromes are followed by a T-rich stretch of residues (TTTATTTT). Although we do not have any experimental data to show where the transcription of the protein A mRNA starts or terminates, it thus appears likely that protein A is translated from a monocistronic mRNA.

Amino Acid Sequence—The amino acid sequence deduced from the DNA sequence as well as amino acids that differ in the partial protein sequence established in Sjödahl (27) are also indicated in Fig. 3. Among the IgG-binding regions D, A, B, and C, a high degree of homology exists and only 4 out of the 235 amino acids comprising all four regions vary. All these changes can be explained by single point mutations. Since the DNA sequence was obtained from strain 8325-4 and the protein sequence from strain Cowan I, the divergence is probably due to strain variation. The partial amino acid sequence of region X also shows high similarity to the deduced sequence although about 10% of the amino acids are different.[1] The amino acid numbering starts with the alanine at nucleotide 292 which has been shown to be the first amino acid of the mature protein A.[2] The stop codon at nucleotide 1,711 thus gives a mature protein A of 473 amino acids and a resulting M_r = 52,752.

Amino Acid Composition—Attempts to determine the protein sequence of protein A have involved digestion of staphylococcal cell walls with lysostaphin (28) or analyzing protein A from mutant bacteria which secrete the product (8). In order to compare the sequences deduced from the DNA sequence with those obtained experimentally, the amino acid compositions of different parts of the protein, as deduced from the DNA sequence, are tabulated in Table I. The amino acid compositions of purified protein A from different strains of *S. aureus* are also presented in Table I. A direct comparison of structures from deduced and purified proteins is difficult, due to strain differences and proteolytic digestion during isolation of the protein. According to Sjödahl (27) and Lindmark *et al.* (8), there are only a few amino acids NH_2-terminal

[2] U. Hellman, unpublished results.

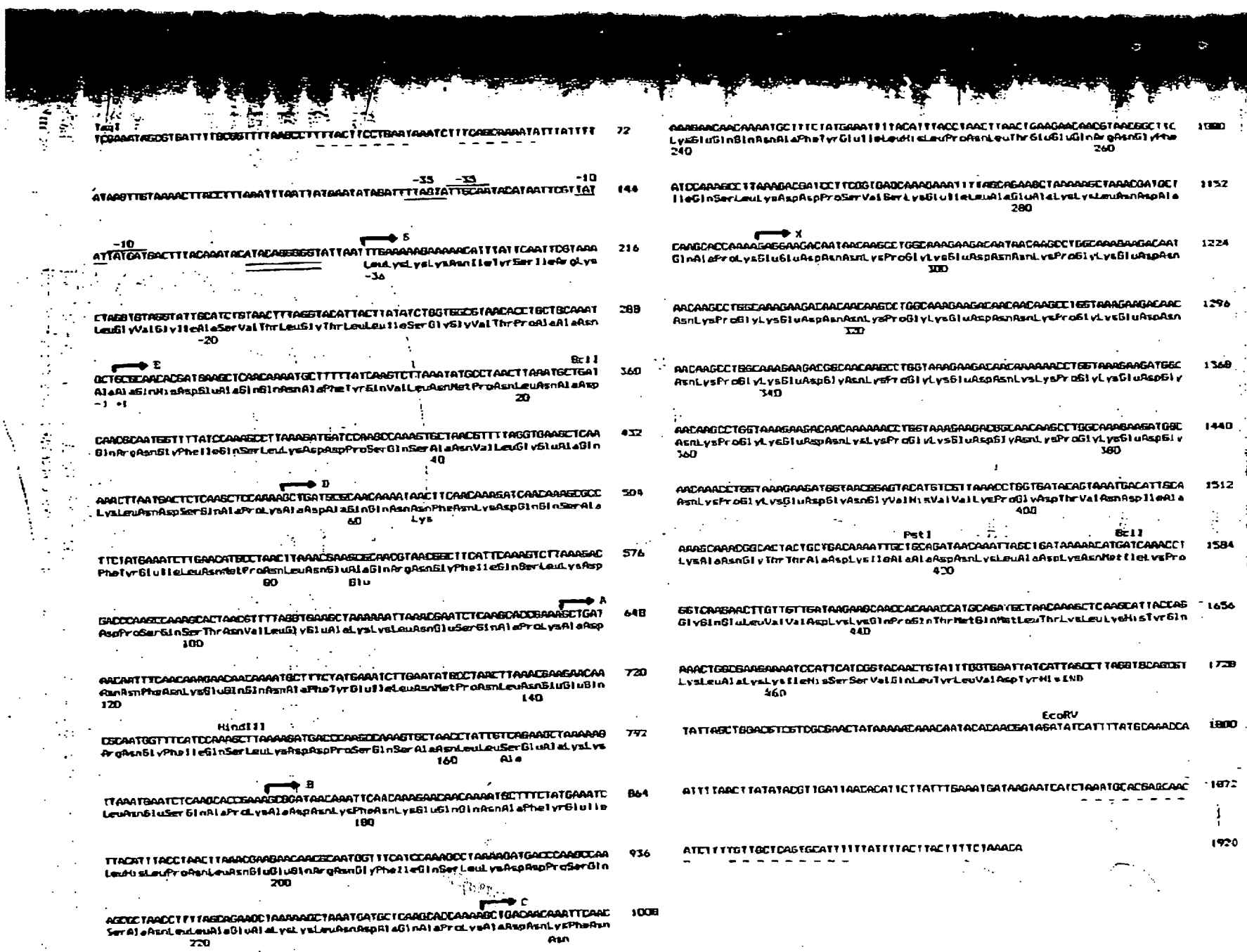

FIG. 3. Nucleotide and amino acid sequence of the protein A gene. Numbering of the amino acids starts at the NH_2 terminus of the mature protein. Two possible promoters (−35 and −10), a possible Shine-Dalgarno (23) sequence (=), and possible transcriptional termination sequences (- - -) are indicated. The amino acids that are different compared to the partial amino acid sequence of Sjödahl (7) (covering amino acids 57–291) are indicated. The starting residues of regions S, E, D, A, B, C, and X are indicated by *arrows*.

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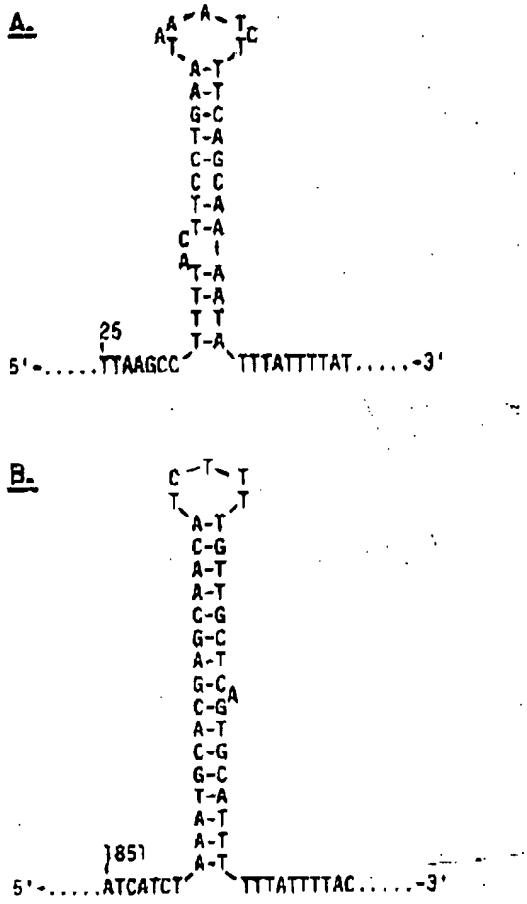

FIG. 4. **Hypothetical secondary structures at the 5′ and 3′ regions flanking the protein A coding sequence.** The *numbers* refer to nucleotides in Fig. 3.

of region D in protein A isolated from cell walls of Cowan I. However, the exact NH_2-terminal sequence could not be obtained due to a blocked terminus (27). Table I shows that the size of the deduced protein from 8325-4 is larger than two independent determinations of the protein from Cowan I even if region E is omitted (A–E). At present, it is unclear if this difference in size and amino acid composition is due to proteolysis both in the NH_2-terminal and COOH-terminal parts of the protein or if it reflects genomic differences. The protein A gene of Cowan I has recently been cloned in our laboratory, which will help to clarify this point.

In contrast, it appears likely that the secreted form of protein A from strain A676 does contain region E. The NH_2-terminal sequence of this protein (8) fits well with the NH_2-terminus of protein A from strain 8325-4 when determined both by Edman degradation of the purified protein[3] and by DNA sequence starting at nucleotide 292 in Fig. 3. The size of protein A from A676 would then indicate that the protein is truncated at the COOH-terminal lacking approximately 80 amino acids. The amino acid composition, as deduced from the DNA sequence, of a mature protein A lacking 107 amino acids in the COOH-terminal part shows good agreement with the composition of purified protein A from strain A676 as shown in Table I. However, the DNA sequence does not contain the COOH-terminal -Val-Ala-Lys which has been reported for A676 (8).

Codon Usage—The codon usage for the preprotein of protein A is compared in Table II with other Gram-positive genes. Chromosomal genes are represented by four *Bacillus*

TABLE I

Amino acid composition of deduced protein A gene or purified protein from different strains of S. aureus

Amino acids	Deduced protein A from 8325-4				Purified protein A		
	Prot-A[a]	Mat-A[b]	A-E[c]	A-X[d]	Cowan I[e]	Cowan I[f]	A676[g]
Lysine	69	65	62	45	52	63	48
Histidine	7	7	6	3	4	4	3
Arginine	8	5	4	5	5	4	4
Aspartic acid	105	103	91	85	82	83	82
Threonine	10	7	7	2	5	6	4
Serine	25	22	18	20	17	16	16
Glutamic acid	78	78	67	68	65	70	64
Proline	31	30	27	24	27	26	27
Glycine	33	28	26	18	30	30	22
Alanine	42	38	31	31	34	36	31
Valine	15	12	10	4	5	8	7
Methionine	6	6	5	3	2	3	3
Isoleucine	18	14	13	10	9	12	11
Leucine	41	36	31	29	27	28	27
Tyrosine	9	8	7	5	5	4	4
Phenylalanine	14	14	12	14	12	12	13
Total	509	473	417	368	381	395	366

[a] Protein A including the signal peptide.
[b] Mature protein A, amino acids 1–473 in Fig. 3.
[c] Mature protein A except region E, amino acids 57–473.
[d] Mature protein A except COOH-terminal part, amino acids 1–366.
[e] From Movitz (2), isolated by lysostaphin treatment of bacteria.
[f] From Lindmark *et al.* (8), isolated by lysostaphin treatment of bacteria.
[g] From Lindmark *et al.* (8), extracellular protein A produced by a methicillin-resistant strain.

genes and plasmid-coded genes by the four putative proteins encoded by the staphylococcal plasmid vector pC194 (26). Also indicated by + or − are the codon pairs which, according to Grosjean and Fiers (33), are most likely to be preferred or not preferred, respectively, by highly expressed genes. Their hypothesis predicts that efficient in-phase translation is facilitated by proper choice of degenerate codewords, and the codon pairs marked in Table II are most dependent on maximal codon-anticodon interaction energy.

Table II shows that among the chromosomal genes the codon usage is randomly distributed. The per cent G/C of the degenerate third base is 42%, similar to the overall GC content of the *Bacillus* species involved, which is 42–47% (34). In contrast, the plasmid-coded genes have a marked preference for A/U bases, only 22% G/C. Although the repetitive nature of the protein A gene makes statistical analysis risky, it seems to exhibit a clear preference for third position A/U bases with a few exceptions, UUC (Phe), AAC (Asn), and AGC (Ser). Two of these exceptions can be explained by the Grosjean and Fiers (32) hypothesis. Furthermore, among the four codon pairs in which, according to the theory, selection for C is preferred, this nucleotide is indeed chosen 64% of the time (67/105). In contrast, the four codon pairs with predicted selection for U show a reversed ratio, and only 21% C (18/85) can be found. The GC content at the third base of the codons is 32%, similar to the GC content of chromosomal DNA from *S. aureus* which is 30–33% (34). Therefore, the codon usage of the protein A gene shows a preference for A/U bases adapting to the overall GC content of the host cell with some exceptions, mainly following the Grosjean-Fiers (33) rules for highly expressed genes.

Homology Plot Analysis—In order to search for homologous regions, the DNA sequence and its deduced amino acid sequence were scanned by a computer program. Every point in

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TABLE II

Codon usage of protein A and other Gram-positive genes

		Prot-A[a]	Chrom[b]	Plasmid[c]	Pref[d]			Prot-A[a]	Chrom[b]	Plasmid[c]	Pref[d]
Phe	UUU	2	45	89	−	Tyr	UAU	8	49	29	−
	UUC	12	20	11	+		UAC	1	33	9	+
Leu	UUA	20	34	35		Term	UAA	0	—	—	
	UUG	5	22	13			UAG	0	—	—	
	CUU	7	31	10		His	CAU	6	27	17	
	CUC	1	7	4			CAC	1	8	1	
	CUA	6	3	5		Gln	CAA	38	33	16	
	CUG	2	31	4			CAG	2	35	6	
Ile	AUU	8	38	27	−	Asn	AAU	20	68	43	−
	AUC	9	30	5	+		AAC	45	31	12	+
	AUA	1	12	18		Lys	AAA	61	79	56	
Met	AUG	6	29	12			AAG	18	28	12	
Val	GUU	5	21	12		Asp	GAU	21	81	22	
	GUC	2	21	1			GAC	19	35	5	
	GUA	6	21	14		Glu	GAA	37	59	19	
	GUG	2	30	4			GAG	1	35	10	
Ser	UCU	5	20	16		Cys	UGU	0	2	7	
	UCC	0	21	1			UGC	0	2	4	
	UCA	3	31	7		Term	UGA	0	—	—	
	UCG	2	22	4		Trp	UGG	0	35	9	
Pro	CCU	21	16	10	+	Arg	CGU	3	18	4	+
	CCC	0	11	5	−		CGC	3	5	1	−
	CCA	8	11	3			CGA	0	10	3	
	CCG	2	25	1			CGG	0	9	0	
Thr	ACU	5	13	14		Ser	AGU	3	19	13	
	ACC	1	16	4			AGC	12	17	3	
	ACA	4	48	15		Arg	AGA	0	11	11	
	ACG	0	45	5			AGG	0	14	4	
Ala	GCU	25	29	9	+	Gly	GGU	18	22	11	+
	GCC	1	36	1	−		GGC	14	36	2	−
	GCA	11	40	6			GGA	1	46	3	
	GCG	5	38	1			GGG	0	20	3	
						Sum		509	1654	655	
						Per cent G/C[e]		32	42	22	

[a] Protein A including the signal peptide (preprotein).

[b] The sum of four *Bacillus* chromosomal genes, *B. amyloliquefaciens* α-amylase (25), *B. subtilis* α-amylase (29), *B. subtilis* SpoOF (30), and *B. licheniformis* penicillinase (31).

[c] Four putative proteins of pC194 (32). As the start codons are yet to be identified, the total open reading frames are taken into account.

[d] The eight codon pairs which are most likely to be preferred (+) or not preferred (−) by highly expressed genes (33).

[e] Per cent G/C in the third degenerate base. The codons AUG (Met), UGG (Trp), and AUA (Ile) are omitted.

the homology plots represents an identical residue (1). The nucleotide triplets and the deduced amino acids are compared in Fig. 5, *A* and *B*, respectively. As the sequence is compared with itself, a line of identity occurs from the left upper corner to the right lower corner, and homologous repeats show up as parallel lines, which disappear when no homology exists. The plots reveal two structurally distinct regions with internal homology, flanked by unique sequences without homology in the 5′ and the 3′ ends of the structural gene. Thus, the part of the gene coding for the signal peptide (*S*) as well as the promoter region (*5′*) seems to be totally unrelated to the IgG-binding regions (*E*, *D*, *A*, *B* and *C*) located in the middle of the gene. The part of the gene coding for the COOH-terminal part of region X as well as the 3′ flanking sequence seems to be unrelated to both the repetitious region X and the IgG-binding regions. Comparisons between the plots show that the homology lines in Fig. 5*A* are more broken than those in Fig. 5*B*, which means that many of the nucleotide changes between the codons in the homologous regions have occurred in bases giving no amino acid change. These results strongly support the previously suggested hypothesis (27) of an evolutionary pressure in these regions keeping the amino acid sequence preserved.

Structure of IgG-binding Regions—The IgG-binding regions of protein A have been defined by trypsin cleavage of the mature protein into functional IgG-binding units D, A, B, and C (7, 27). Recently, we showed (10) that strain 8325-4 also contains a fifth region E homologous to the four repetitive regions earlier identified by protein sequencing. In Fig. 6 the sequence of the regions are aligned to enable comparisons. In order to achieve maximal homology, the boundary of these regions has been moved 15 nucleotides towards the 3′ end of the gene. This choice is of course arbitrary as the 5′ end and the 3′ end of the repetitive region have diverged slightly. However, although the last five amino acids of region C′ (292–296) are changed compared to region B′, more than half of the nucleotides (8/15) are homologous, indicating a relationship. The same holds for the other end of the repetitive region located in the beginning of region E′. Although the first three amino acids are different from region D′, five out of nine nucleotides are identical. The cleavage points for trypsin are marked with *arrows*. There exists a nine-nucleotide insertion in region E′ giving three amino acid residues (59–61) not homologous to the other regions. Also shown in Fig. 6 are the sequences flanking the repetitive regions. As already pointed out in the homology analysis (Fig. 5, *A* and *B*) these regions seem to be nonhomologous to the IgG-binding regions.

A changed nucleotide compared to region B′ in Fig. 6 is

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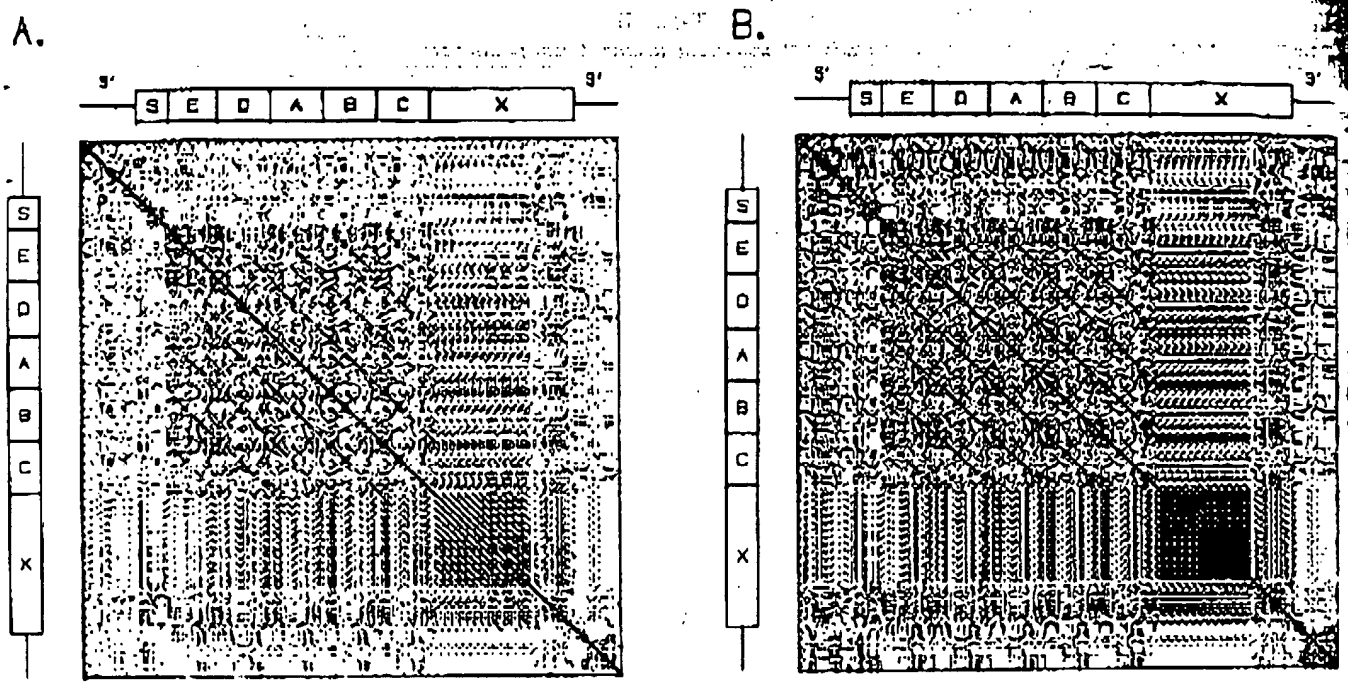

FIG. 5. **Dot matrix comparisons of the protein A sequence.** *A*, the entire nucleotide sequence and the immediate 5' and 3' flanking sequences are compared with itself. Each *dot* represents the center of a three-base identity, and direct repeats appear as *parallel lines* across the *grid*. *B*, the deduced amino acid sequence compared with itself.

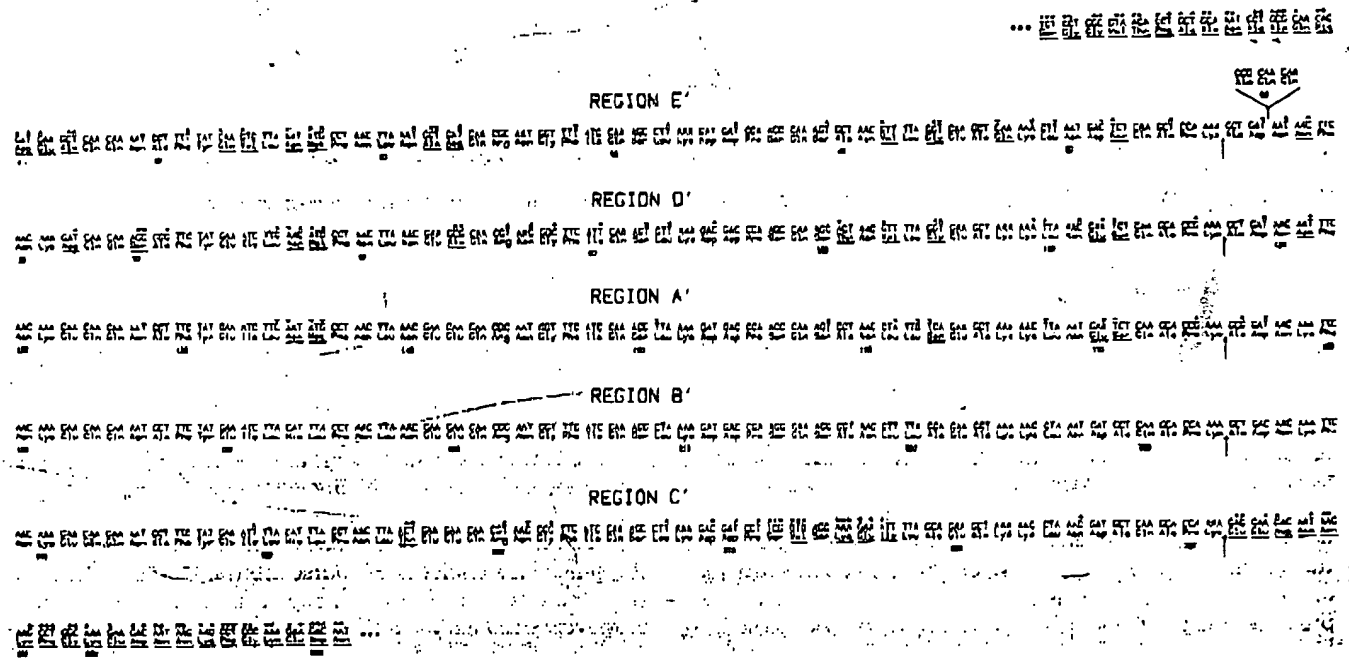

FIG. 6. **Comparisons of the IgG-binding regions and flanking regions.** The sequences of the repetitive regions have been aligned to achieve maximal homology. The comparison is based on region B', and a nucleotide is marked with an *asterisk* and an amino acid is *underlined* when different from the B' region. The cleavage points for trypsin are marked with *arrows*.

marked with an *asterisk*, and a changed amino acid is *underlined*. Table III summarizes the amino acid changes and Table IV the codon changes between the regions. A comparison of the five regions with respect to mutual relationship reveals a pronounced "homology gradient" along the protein molecule, i.e. the closer the location of two regions, the higher the degree of homology. As already pointed out by Sjödahl (27), one structural gene coding for the IgG-binding part of protein A has been subjected to stepwise gene duplications involving only one region followed by a period in which point mutations have occurred, thus generating slightly dissimilar nucleotide and amino acid sequences. As a result of these evolutionary events, a homology gradient will evolve. The fact that codons (Table IV) have changed much faster than amino acids (Table

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Exhibit 2

BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS 248, 315–323 (1998)
ARTICLE NO. RC988922

Cell-Specific Transfection of Choriocarcinoma Cells by Using Sindbis Virus hCG Expressing Chimeric Vector

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Received May 31, 1998

The development of Sindbis virus vectors that can target specific cell types would provide an important gene therapy strategy. We explored the possibility of designing a Sindbis virus vector that can target human choriocarcinoma cells via ligand-receptor interaction. The Sindbis virus envelope gene was modified by insertion of the α- and β-hCG genes. The chimeric helper RNA was then transfected into BHK cells along with a virus-based expression vector, allowing the production of virus particles containing hCG-envelope chimeras. The hCG-envelope chimeric virus vector has minimal infectivities against BHK cells and human cancer cells which do not contain LH/CG receptors on their surface. This vector can, however, infect and transfer a reporter gene to choriocarcinoma cells as well as other cells bearing LH/CG receptors. This chimeric Sindbis virus vector may provide a novel approach for gene therapy of gestational trophoblast disease and placental dysfunction. © 1998 Academic Press

Choriocarcinoma is one type of gestational trophoblastic disease and malignant transformation of molar tissue or a de novo lesion arising spontaneously from the placenta of a term pregnancy or spontaneous abortion. This disease usually progresses rapidly and, without treatment, metastatic disease is frequently fatal, as choriocarcinoma cells readily invade blood vessels, producing metastasis via hematogenous routes of dissemination. The most frequent metastatic region is the lungs(1, 2) which is classified as FIGO stage III, followed by liver(3, 4) and brain(5, 6) (FIGO stage IV) (7). Basically, management of the original tumor is surgical extirpation and chemotherapy. However, for metastatic tumors, especially metastasis to liver and brain (high-risk matastasis), multiple-agent chemotherapy is recommended(3, 6, 8). Metastatic tumors are difficult to treat and there is a limit to chemotherapy with respect to tolerance and side effects. Moreover, because patients with high-risk metastatic choriocarcinoma who have failed primary chemotherapy have a very poor prognosis, it is clear that novel therapeutic approaches will be required to significantly improve survival rate of patients with metastasis.

Although various systems of gene therapy for cancer have been developed, including virus-based expression vectors(9-12), most of them did not have the ability to target specific cells. Several attempts to alter the host range of viruses have been reported. For retrovirus-based vectors, direct modifications of the envelope protein of murine leukemia virus (MLV) have been shown to redirect the viral binding activity or tropism. A recombinant virus containing a fragment encoding a single Fv antibody chain at the N terminal region of the MLV *env* gene has been shown to recognize the corresponding epitopes (13) and infect human cells (14, 15). Kasahara *et al.* have made a chimeric ecotropic virus containing an erythropoietin-envelope fusion protein (16). This chimeric virus has been shown to infect human cells bearing the erythropoietin receptor. They also have produced the ecotropic virus envelope modified by insertion of human heregulin and this chimeric virus can infect human breast cancer cell lines that overexpress the human epidermal growth factor receptor (17). In these systems, although retroviruses have the advantage of mediating stable gene transfer with a low potential for immunogenicity, there are some problems with respect to therapeutic use including: difficulties in producing high titers of retrovirus, the fact they can infect only actively dividing cells, and the possibility of insertional mutagenesis(18). There is a report of targeted gene delivery by tropism-modified adenoviral vectors, which can bind with a target receptor on the cell surface when they are complexed with conjugation of neutralizing antibody directed against the knob domain of the fiber capsid protein and cell-specific ligand(19). However, the adenovirus vector system, while capable of delivering genes with high efficiency

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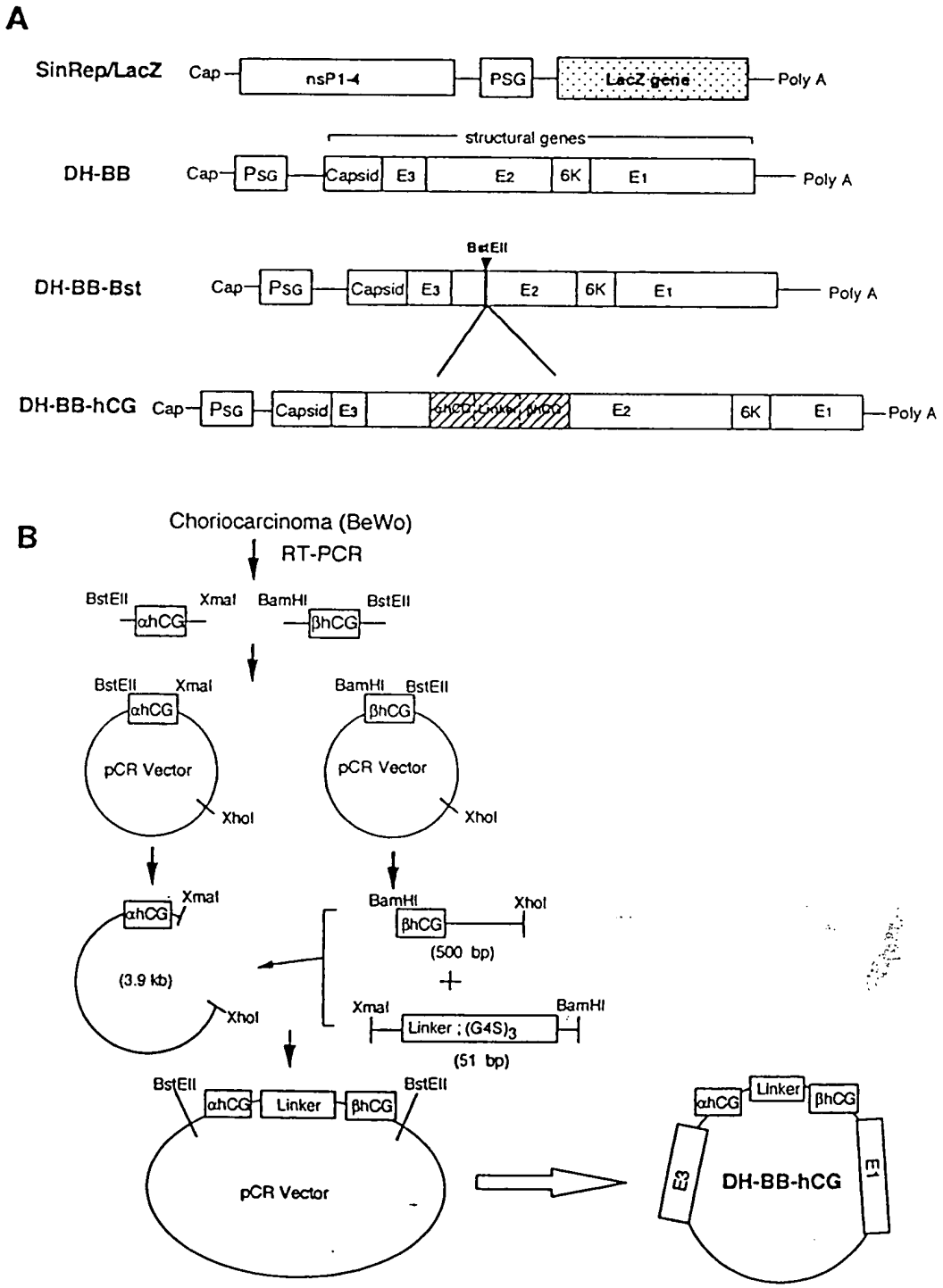

FIG. 1. (A) Schematic representation of SinRep/LacZ expression vector and recombinant helper constructs. SinRep/LacZ is a Sindbis virus-based expression vector which contains the packaging signal, nonstructural protein genes for replicating the RNA transcript, and the lacZ gene as a reporter gene. DH-BB is a parental helper plasmid which contains the genes for the structural proteins (capsid, E3, E2, 6K, and E1) required for packaging of the Sindbis viral genome. DH-BB-Bst was constructed by introduction of a cloning site (BstEII) into the E2 protein between amino acids 71 and 74. The synthetic α and β hCG genes were inserted at BstEII in the DH-BB-Bst helper plasmid and DH-BB-hCG was obtained. Abbreviations: P_{SG}, Sindbis viral subgenomic promoter; nsP1-4, nonstructural protein genes 1-4; Poly A, polyadenylation signal. (B) Construction of recombinant DH-BB-hCG helper. Construction protocol of the recombinant plasmid is described schematically. The details are included under Materials and Methods. The regions of α and β hCG genes were obtained from RT-PCR of total choriocarcinoma cell RNA and they were inserted into the pCR vector. The α and β hCG genes were cloned into the pCR vector along with a $(G_4S)_3$ linker and then the synthetic hCG gene was inserted into DH-BB-Bst plasmid to create DH-BB-hCG.

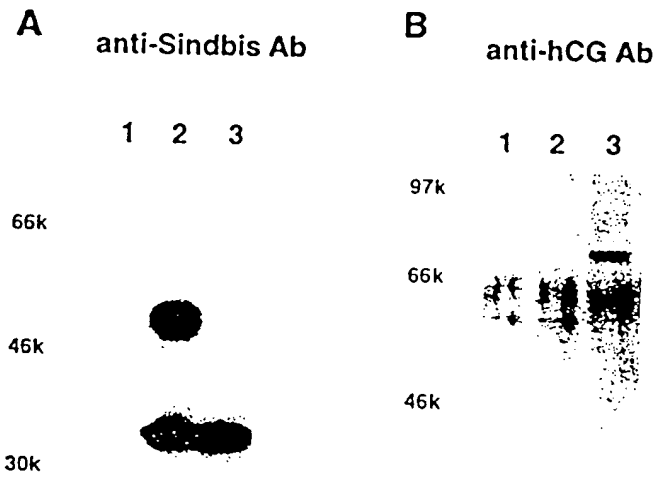

FIG. 2. Detection of Sindbis viral structural components and a recombinant envelope. Pellets of viral particles produced from BHK cells transfected with helper (DH-BB or DH-BB-hCG) RNA and SinRep/lacZ RNA were subjected to SDS-PAGE analysis. After transferring to a nitrocellulose filter, viral proteins were stained with anti-Sindbis virus mouse immune ascitic fluid to detect all structural components (A) or with anti-hCG rabbit whole antiserum to detect hCG-envelope chimeric protein (B). In each panel: lane 1, control supernatants of BHK cell; lane2, DH-BB; lane3, DH-BB-hCG.

Infection assay with viruses bearing chimeric envelope. Infectivities of recombinant viruses against hamster and human cells were determined by transfer of the Sindbis virus vector (SinRep/LacZ) that can transduce the bacterial β-galactosidase gene. Viruses derived from the DH-BB helper showed very high infectious titer (10^8 LacZ CFU/ml) against BHKcells, whereas viruses produced by DH-BB-hCG showed very low infectivity (10^2 LacZ CFU/ml). Insertion of the α- and β- hCG heterodimer into the E2 protein may block virus binding to host cells as suggested by previous observations (38).

As shown in Figure 3, where infected and uninfected cells were stained by X-gal to estimate tropism of the hCG-envelope chimeric virus, viruses derived from the DH-BB-hCG helper can infect the choriocarcinoma cell line, BeWo, which expresses LH/CG cell surface receptors (31). The level of infectivity is equivalent to that of viruses derived from DH-BB helper RNA. Although viruses derived from the DH-BB helper can infect both the epitheloid carcinoma cell line, HeLaCD4+, and the epidermoid carcinoma cell line, A431, which do not express the hCG receptor, the cell lines cannot be infected by the hCG-envelope chimeric virus. Moreover, the glioblastoma cell line, U87MG, was infected with hCG-envelope chimeric Sindbis viruses, because these cells, which produce hCG glycoprotein(39) and are derived from brain cells bearing LH/CG receptors(40), should have LH/CG receptors on their surface. To verify this hypothesis, we assayed ^{125}I-hCG binding(41) to U87MG cells. U87MG cells were found to bind ^{125}I-hCG as well as choriocarcinoma cells, indicating the presence of LH/CG receptors (data not shown). In addition, when BeWo cells and U87MG cells were preincubated with an excess amounts of hCG (100 IU/ml) for 15 min before infection, these cells did not stain with X-gal, as was the case for negative control groups (data not shown).

Next, we measured total β-gal amounts of various cells (2×10^5) infected with viruses derived from DH-BB-hCG helper by using the CPRG β-gal assay. As shown in Figure 4A, after infection of hCG-envelope chimeric Sindbis viruses, BeWo and U87MG cells, which have LH/CG receptors on their surface, expressed much higher β-gal amounts than HeLaCD4+ and A431 cells. BeWo andU87MG cells also expressed higher β-gal amounts than basal levels before infection($p < 0.005$), whereas HeLaCD4+ and A431 cells expressed similar β-gal amounts before and after infection. Other choriocarcinoma cell lines, JAR and JEG-3, were also susceptible to infection with viruses derived from DH-BB-hCG helper and expressed higher β-gal amounts after infection(data not shown). Figure 4B shows the dose-dependent effect of hCG-envelope chimeric viruses on the lacZ gene transfection to the choriocarcinoma cell line, BeWo. We utilized chimeric virus from 100μl to 2ml to infect 2×10^5 cells and within this concentration range, this virus vector could transfect the lacZ gene in a dose-dependent manner. Figure 4A and B indicate that viruses derived from DH-BB-hCG helper can infect LH/CG receptor bearing cells and transfect the lacZ reporter gene to cells with high efficiency.

Cytotoxic effect. Several studies have shown that Sindbis virus infection of vertebrate cells usually results in apoptosis both *in vitro* (42, 43) and *in vivo*(44). When a Sindbis virus vector has the ability to specifically infect cancer cells, this cytotoxicity may be suitable for gene therapy of cancer. To estimate the cell specific cytotoxicity of viruses derived from the DH-BB-hCG helper, we determined cancer cell proliferation by the XTT assay. XTT works by being metabolized by mitochondrial dehydrogenases to form a formazan dye and then the amount of formazan dye formed directly correlates to the number of metabolically active cells in the culture. As expected, viruses derived from DH-BB-hCG helper did not reduce cell proliferation of HeLaCD4+ (Figure 5A), because these viruses can not infect HeLaCD4+ cells (Figure 3 and 4). Meanwhile hCG-envelope chimeric viruses reduced cell proliferation of BeWo to 68% of that observed in uninfected cells. As compared with viruses produced from DH-BB helper, hCG-envelope chimeric viruses reduced cell proliferation of infected choriocarcinoma cells, JEG-3, on both day 2 and day 3 after infection to a lesser extent (Figure 5B).

Effect of 8-Br-cAMP on infectivity of hCG-envelope chimeric virus. One of the choriocarcinoma cell lines,

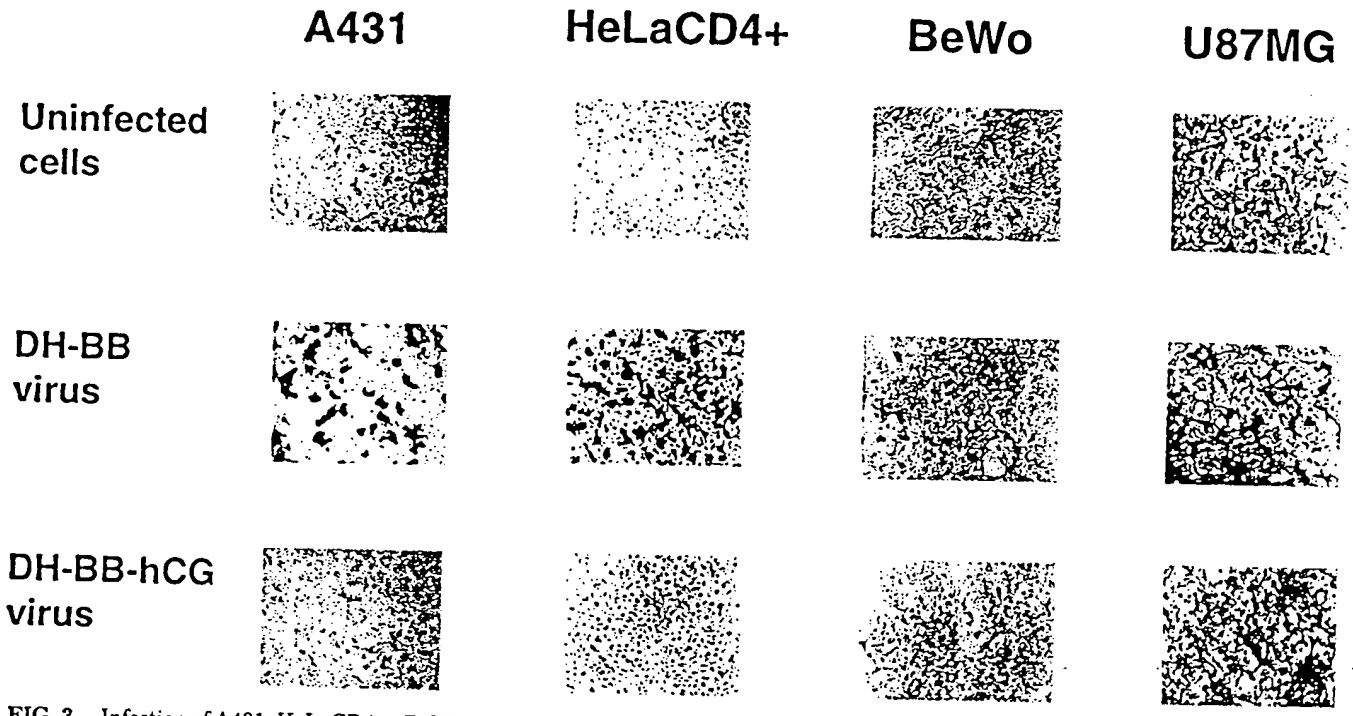

FIG. 3. Infection of A431, HeLaCD4+, BeWo, and U87MG cells with Sindbis virus produced from DH-BB helper RNA and recombinant Sindbis virus derived from DH-BB-hCG helper RNA which transduce the bacterial lacZ gene. After washing with PBS, viral supernatants (50 μl of DH-BB, 1ml of DH-BB-hCG) were added to 2×10^5 cells of each type plated in 6-well plates. After 1 hr incubation at room temperature, cells were washed with PBS again and incubated in growth medium for 24 hr. Viral infectivity was evaluated by X-gal staining.

BeWo, undergoes cellular differentiation when stimulated by cAMP. In the differentiated state, the cells fuse to form syncytia and have been shown to resist adenovirus infection(45) in the presence 8-Br-cAMP. To examine whether hCG-envelope chimeric Sindbis virus infection is also inhibited by cellular differentiation, we estimated total β-gal amounts of differentiated BeWo cells, preincubated with 8-Br-cAMP, after chimeric Sindbis virus infection. As shown in Table I, JEG-3 cells, which are not induced by 8-Br-cAMP to form syncytia, did not show a reduced infection efficiency of hCG-envelope chimeric Sindbis viruses upon 8-Br-cAMP exposure. Differentiation of BeWo cells by 8-Br-cAMP also did not result in a reduced infection efficiency (3.5 ± 0.5 ng of total β-gal amounts) of Sindbis virus derived from DH-BB-hCG helper as compared with total β-gal expression of undifferentiated cells infected by hCG-envelope chimeric viruses (3.8 ± 0.6 ng of total β-gal amounts). Unlike the adenovirus system, these observations demonstrate the ability of the Sindbis virus system to infect both differentiated and undifferentiated cells.

DISCUSSION

Although considered a useful virus-derived vector system, Sindbis virus vectors present a cell targeting problem in that they naturally infect most cells. For mammalian cells, the high affinity laminin receptor was identified as one of the Sindbis virus receptors. The wide distribution and highly conserved nature of the laminin receptor may be responsible for the broad host range of this virus (28, 46). In this study, we developed a recombinant Sindbis virus vector displaying hCG-envelope chimeric proteins on the viral surface and demonstrated that this chimeric virus vector has the tropism to permit gene delivery specifically to target cells bearing LH/CG receptors. In the mature Sindbis virus virion, a plus-stranded viral genome RNA is complexed with capsid protein C to form an icosahedral nucleocapsid that is surrounded by a lipid bilayer in which two integral membrane glycoprotein, E1 and E2, are embedded(28). In this study, however, the incorporation of E1 glycoprotein and hCG-E2 chimeric glycoprotein into virions could not be detected by anti-Sindbis antisera, although we could detect a hCG-E2 chimeric viral envelope glycoprotein by anti-hCG antisera. Insertion of the hCG glycoprotein may produce a structural change of the recombinant E2 chimeric protein that inhibits binding with the anti-Sindbis antisera. From other studies (unpublished data), it appears that the E1 protein is present in the recombinant virion but at remarkably reduced levels.

The hCG-envelope chimeric Sindbis virus vector showed minimal infectivities against BHK cells and some human cancer cell lines which lack the LH/CG receptor on their surface. On the other hand, this hCG-

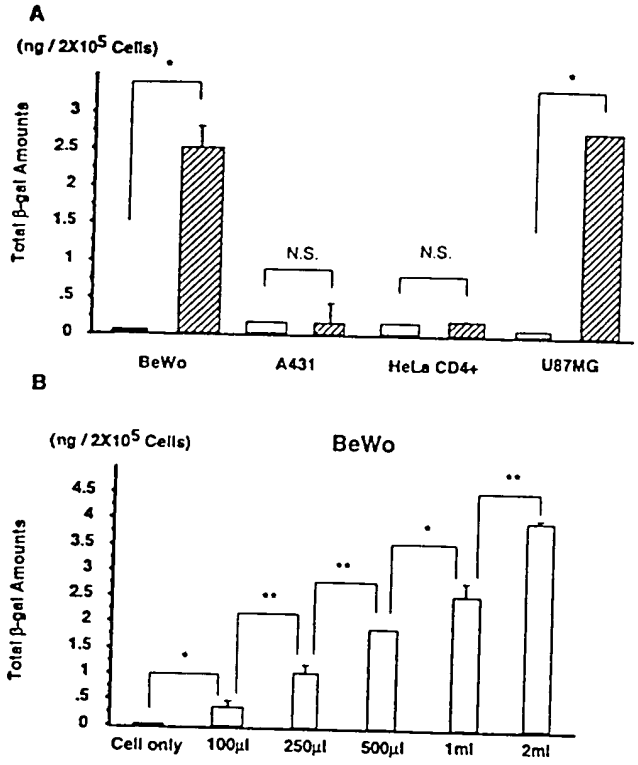

FIG. 4. (A) Total β-gal amounts of BeWo, A431, HeLaCD4+, and U87MG cells (2×10^5) after infection of 1 ml of viral supernatant derived from DH-BB-hCG(shaded bars). Twenty-four hours after infection, cytoplasmic β-gal levels were detected by CPRG assay (Materials and Methods). Blank bars represent total β-gal amounts in uninfected cells. Values are the means±SEM of three independent experiments. *, $p<0.005$; N.S., not significant. (B) Dose-dependent effect of hCG-envelope chimeric Sindbis virus on the total β-gal expression in BeWo cells (2×10^5). Cells were infected with viral supernatant and, 24 hr after infection, β-gal expression was measured by CPRG assay. Values for each are the means±SEM of three independent experiments. *, $p<0.05$; **, $p<0.005$.

envelope chimeric virus could infect LH/CG receptor-bearing cancer cell lines and transfer the lacZ gene into these cells with high efficiency in a viral dose-dependent manner. A previous study reported that choriocarcinoma contained more LH/CG receptor mRNA and protein than hydatidiform moles which in turn contained more than normal human placenta(31). Therefore, hCG-envelope chimeric virus vector could be valuable for gene therapy of gestational trophoblastic disease, especially for choriocarcinoma. The hCG-chimeric virus was also shown to cause cytotoxicity to infected cells at comparable levels with wild-type Sindbis virus (42-44). This cytotoxicity may be suitable for gene therapy of choriocarcinoma cells.

In this study, we have demonstrated that this hCG-envelope chimeric virus vector may be useful to gene therapy for, not only choriocarcinoma, but also glioblastoma, which have LH/CG receptors on their surface. Recently, overexpression of LH/CG receptor at both mRNA and protein levels has been reported in endometrial carcinoma(47). Some types of ovarian cancer, endometrioid, clear cell carcinoma and mixed subtype, most likely also overexpress LH/CG receptors because these ovarian cancer cells are pathologically common in neoplasms arising from endometriosis(48) which have increased expression of LH/CG receptor(49, 50). Accordingly, the hCG-envelope chimeric virus vector may be able to infect and transfect genes to endometrial carcinoma and some kinds of ovarian cancer.

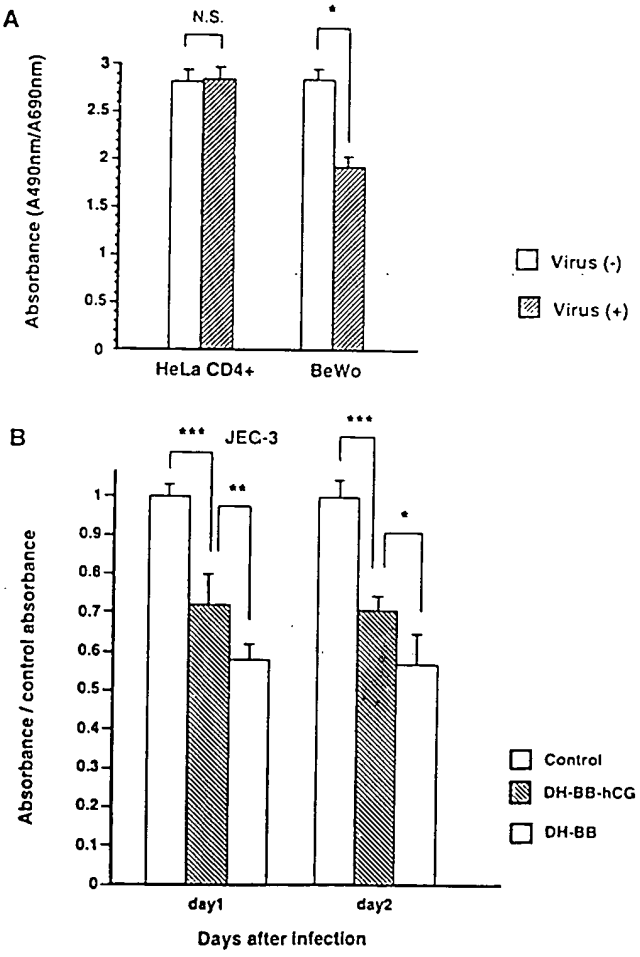

FIG. 5. (A) Cytotoxic activity of Sindbis viruses derived from DH-BB-hCG helper. HeLaCD4+ and BeWo cells were infected with 50 μl of hCG-envelope chimeric virus in 96-well plates and, 24 hr after infection, infected cells and uninfected cells were assayed for cell proliferation as described under Materials and Methods. Values for each are the means±SEM of three independent experiments. *, $p<0.01$; N.S., not significant. (B) Cytotoxic activity of viruses derived from DH-BB-hCG helper and DH-BB helper. Choriocarcinoma, JEG-3, cells were infected with both viruses (50 μl of DH-BB-hCG, 1 μl of DH-BB) in 96-well plates. One or 2 days after infection, infected cells and uninfected cells were assayed for cell proliferation (Materials and Methods). Figure scales represent the absorbance ratio of experimental to control groups (uninfected cells). Values for each are the means±SEM of three independent experiments. *, $p<0.05$; **, $p<0.01$; ***, $p<0.005$.

TABLE I

Effect of 8-Br-cAMP on Infectivity of hCG-Envelope Chimeric Virus

	Preincubation with 8-Br-cAMP	Virus (−)	DH-BB-hCG virus
JEG-3	−	0	4.0 ± 0.6 ng
	+	0	3.8 ± 0.7 ng
BeWo	−	0.2 ng	3.8 ± 0.6 ng
	+	0.2 ± 0.1 ng	3.5 ± 0.5 ng

Note. Total β-gal amounts of JEG-3 and BeWo cells (2×10^5 cells), with or without preincubation of 8-Br-cAMP, after infection of hCG-envelope chimeric Sindbis virus. Cells were preincubated with 1.5 mM of 8-Br-cAMP for 16 hr or not, and infected with 1 ml of hCG-envelope chimeric virus or not, and then, 24 hr after infection, β-gal amounts were detected by CPRG assay. Values for each are the means ± SEM in three independent experiments.

As regards clinical applications, this hCG-envelope chimeric Sindbis virus vector may not be able to be administered systemically by the intravenous route yet, mainly because of problems such as inadequacy of virus titers. This virus vector, however, can serve as a useful model system for examination of targeted gene transfer in cancer therapy and several potential applications of cell-specific Sindbis vectors can be proposed. The cancer-specific virus vector, for example, could deliver the herpes thymidine kinase gene(12), bacterial cytosine deaminase gene(51) or cytokine genes to kill cancer cells with ganciclovir or 5-fluorocytosine treatment or to induce a cytotoxic T-cell response to the cancer cells, respectively.

It is natural that we think this hCG-envelope chimeric virus vector will be useful for gene transfer to normal placenta. The placenta plays an important role in supporting fetal growth and placental insufficiency has severe consequences for the health of mother and fetus(53-55). The development of gene therapy of the placenta could provide a novel therapy of placental dysfunction. Sindbis virus vector derived from the DH-BB-hCG helper can infect not only undifferentiated BeWo cells but also differentiated BeWo cells, which includes cell fusion to form syncytia by cAMP-stimulation in this study (Table I), in contrast to adenovirus vectors which cannot infect differentiated choriocarcinoma cells and trophoblasts(45). Moreover, LH/CG receptors of normal trophoblasts can bind more hCG glycoprotein than the receptors of choriocarcinoma cells (56). The hCG-envelope chimeric Sindbis virus should, therefore, be able to infect normal placenta, which is constructed with undifferentiated cytotrophoblasts and differentiated syncytiotrophoblasts.

Considering that Sindbis virus infection of vertebrate cells usually results in cell death by apoptosis (43) as described above, utilization of hCG-envelope chimeric virus for gene therapy of normal organs will require further modifications of the virus. There are some reports that the transformation of cells with the cellular oncogene *bcl-2* led to a cell line in which Sindbis virus no longer induces apoptosis and instead establishes a persistent infection(43, 57). In addition, a single coding mutation in the nonstructural protein gene produced a virus which can establish persistent infection (58). We are currently investigating the possibilities of utilizing bcl-2 to construct a long-term Sindbis virus expression vector and the generation of a mutated Sindbis virus vector which does not induce apoptosis.

In this study, we developed a hCG-envelope chimeric Sindbis virus vector that can target cancer cells which express LH/CG receptors. The development of Sindbis virus vectors that can target malignant cells overexpressing specific cell surface proteins would have important implications for the gene therapy of cancer.

ACKNOWLEDGMENTS

This work was supported by National Institutes of Health Grants CA68498 and CA22247. The authors are grateful to Dr. Christine Pampeno for critically reading the manuscript.

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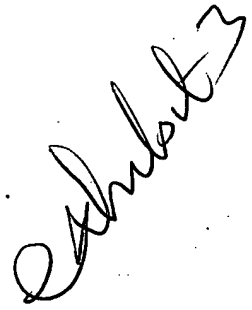

Proc. Natl. Acad. Sci. USA
Vol. 96, pp. 4005–4010, March 1999
Medical Sciences

Identification of the block in targeted retroviral-mediated gene transfer

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Communicated by Inder M. Verma, The Salk Institute for Biological Studies, San Diego, CA, December 24, 1998 (received for review March 31, 1998)

ABSTRACT **A chimeric retroviral vector (33E67) containing a CD33-specific single-chain antibody was generated in an attempt to target cells displaying the CD33 surface antigen. The chimeric envelope protein was translated, processed, and incorporated into viral particles as efficiently as wild-type envelope protein. The viral particles carrying the 33E67 envelope protein could bind efficiently to the CD33 receptor on target cells and were internalized, but no gene transfer occurred. A unique experimental approach was used to examine the basis for this postbinding block. Our data indicate that the chimeric envelope protein itself cannot participate in the fusion process, the most reasonable explanation being that this chimeric protein cannot undergo the appropriate conformational change that is thought to be triggered by receptor binding, a suggested prerequisite to subsequent fusion and core entry. These results indicate that the block to gene transfer in this system, and probably in most of the current chimeric retroviral vectors to date, is the inability of the chimeric envelope protein to undergo this obligatory conformational change.**

A major goal of gene therapy research is to develop vectors that would allow targeted gene transfer into specific cell types (1). Several attempts have been made either to substitute or to insert a ligand (either a peptide or a single-chain antibody) into the envelope protein of a retroviral vector so that the vector could then bind to a specific receptor on a designated cell type (2–14). In initial studies, antibodies were used to bridge the vector and the host cells (3, 4). Because of the low efficiency, more recent studies have engineered the envelope protein in an attempt to change the tropism of the retroviral vector. A ligand to the erythropoietin receptor or to the heregulin receptor has been used to replace the binding domain of the murine leukemia virus (MuLV) ecotropic envelope protein to achieve transduction of target cells (5, 6). Insertion of a single-chain antibody (scFv) or a ligand into the N-terminal region of the envelope protein also has been used to target cell-surface molecules (7–12). In addition to the ecotropic Moloney murine leukemia virus (Mo-MuLV), the envelope protein of spleen necrosis virus has been used as a model system (13, 14). However, although some of these studies report individual clones that reach a titer as high as 10^4 on target cells, it has not been possible to reliably generate vector preparations carrying chimeric envelope proteins that are able to produce titers higher than a few hundred on target cells. A number of laboratories have tested alternative insertion and replacement constructs with different single-chain antibodies and ligands. A significant titer on target cells has not been consistently achieved despite the ability of these chimeras to specifically bind to the target cells.

To identify the basis for this failure, we examined each of the steps in the gene transfer pathway (binding, internalization, fusion, core entry, reverse transcription, integration, and gene expression) to determine the cause of the block. The data suggested that a postbinding block to fusion existed. We then developed a system that allowed us to test, via genetic complementation, individual steps in the fusion process. Even though direct evidence has not been obtained for the exact mechanism for viral fusion in Mo-MuLV, by analogy to other viruses it is thought that, after binding to receptor, Mo-MuLV envelope protein undergoes a conformational change that leads to fusion and core entry. Our data suggest that it is this conformational change that cannot occur in the chimeric envelope protein.

MATERIALS AND METHODS

Envelope Proteins and Cell Lines. A single-chain antibody to human CD33 (15) was constructed by splicing PCR as described (16). Mo-MuLV envelope protein expression vector wild-type ecotropic envelope protein (CEE+) (17) was engineered to contain *Sfi*I (5′ end) and *Not*I (3′ end) sites between amino acids 6 and 7 of the mature envelope protein as described (10). The scFv of CD33 was amplified by PCR with primers (5′-GCCCGGGGGCCCAGCCGGCCATGCACC and 5′-GCG-GTGGCGGCCGCGGAAAGGGTGACC), and the product was subcloned into the *Sfi*I and *Not*I sites of the modified envelope protein to produce the chimeric envelope 33E67. The same approach was applied to the binding-defective mutant D84K (17) to obtain the construct 33K67. The corresponding constructs in the R-peptide truncated form were obtained by subcloning 33E67 and 33K67 into the R-less form of the Mo-MuLV envelope in the construct CEETR (18). Both NIH 3T3 and 3T3/CD33 cells [NIH 3T3 cells that stably express human CD33 (19)] were grown in DMEM (Core Facility, University of Southern California) supplemented with 10% fetal calf serum (FCS, HyClone) and 2 mM glutamine (BRL).

Retroviral Vector Production and Characterization. Retroviral vectors were produced by transient transfection of 293T cells by calcium phosphate precipitation, essentially as described (20, 21). The plasmids used were pHIT60, a Mo-MuLV *gag-pol* expression plasmid, the retroviral vector pCnBg that expresses the *lacZ* and neo genes (21), and one or two envelope expression plasmids. Thirty-six hours after transfection, the supernatants were harvested and filtered through a 0.45-μm filter. The protein content of virions partially purified through 20% sucrose was assessed by Western blot analysis as described (22). The ability of virions to bind to the ecotropic receptor expressed on NIH 3T3 or to the human CD33 antigen expressed on 3T3/CD33 cells was determined by a fluorescence-activated cell sorting assay as described (23). The transduction efficiency of the vector on target cells was determined by titer as described (22). To measure syncytia formation in NIH 3T3 or 3T3/CD33 cell monolayers, 2×10^5 cells were plated in a

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Abbreviations: MuLV, murine leukemia virus; Mo-MuLV, Moloney murine leukemia virus; EM, electron microscopy; SU, surface protein; TM, transmembrane protein; CEE+, wild-type ecotropic envelope protein.

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60-mm tissue culture dish and transfected with 1- or 2-envelope DNA plasmids as described (22). After staining with methylene blue, cells with more than four nuclei were scored as syncytia under phase-contrast microscopy. To measure the formation of heterooligomers, two envelope constructs were cotransfected into 293T cells and coimmunoprecipitation was performed as described (22).

Detection of Viral cDNA and Internalization Assays. Retroviral vector supernatants from cultures of transfected cells were applied to NIH 3T3 or 3T3/CD33 cells at multiplicity of infection = 1 for CEE+ (or an equivalent volume of supernatant for chimeric virus) and incubated for 6 hr at 37°C. Cells were then lysed and the cytoplasmic DNA purified as described (24). The DNA was analyzed by Southern blotting with a ^{32}P-labeled probe containing a long terminal repeat sequence (24). To detect the internalization of vector, ^{35}S-labeled viral particles were incubated with NIH 3T3 or 3T3/CD33 cells for 2 hr at 4°C to allow the virus to bind with its receptor; after washing with cold PBS, the cells were incubated at 37°C for 45 min. At the end of the incubation, the cells were trypsinized at 37°C for 10 min, neutralized with regular D10, washed three times with PBS, lysed, and then immunoprecipitated as described (22).

For electron microscopy (EM) studies, chimeric or wild-type retroviral vector particles were applied to 3T3/CD33 or 3T3 cells and incubated at 37°C for 1 hr. After trypsinization, target cells were collected, washed, and centrifuged for fixation. EM samples were fixed in half-strength Karnovsky fixative for 2 hr then postfixed in 1% osmium tetroxide for 1 hr. After fixation, the samples were dehydrated in a graded series of ethanol/water dilutions, infiltrated with an epon araldite resin mixture, and subsequently embedded in epon araldite resin. Thin sections (60 to 80 nm) were cut and counterstained with uranyl acetate and lead citrate. Viral particles located within the cytoplasm of 12 to 24 randomly chosen cells were counted from each sample on a Zeiss EM 10 electron microscope. Data from two individual experiments were combined for statistical analysis.

RESULTS AND DISCUSSION

Construction of Chimeric Retroviral Envelope Proteins. We analyzed over 30 insertion sites throughout the N-terminal half of surface protein (SU) (B. Wu and W.F.A., unpublished work). Many of these sites were based on examination of the published three-dimensional structure. Of those insertion sites (around 10) that resulted in envelope incorporation into virions as well as at least some binding, an insertion site between amino acids 6 and 7 was chosen for detailed study because this particular insertion site in the envelope protein appears to be the one most receptive to a large insertion (7–10). The CD33 chimera was selected for detailed analysis because it is incorporated into viral particles at a wild-type level, and it has a high binding affinity. However, studies were also carried out with CD34 (42), her2, her4, and several other ligands with similiar results to those obtained for CD33.

We inserted a single-chain antibody to human CD33 (15) into the N-terminal region between amino acids 6 and 7 of the envelope protein of Mo-MuLV to yield the chimeric envelope, 33E67. We then constructed a series of modified chimeric envelope proteins to allow testing of the various steps in the gene transfer process. The chimeric envelope proteins used in this study are shown in Fig. 1. The constructs are 33E67, in which a scFv to the cell-surface antigen CD33 was inserted between amino acids 6 and 7 in the ecotropic envelope (E) protein SU; 33K67, in which a point mutation, D84K (17), was made in the natural receptor binding domain of 33E67 (this mutation prevents binding to the ecotropic receptor, MCAT-1); 33E67TR, in which the 33E67 envelope protein is truncated at the R-peptide cleavage site; and 33K67TR, which is the complement to 33E67TR, in which the 33K67 envelope protein is truncated at the R-peptide cleavage site. Truncation of the R-peptide significantly enhances the fusogenicity of the envelope protein in NIH 3T3 cell syncytia assays (25, 26). The chimera 33K67 was constructed to differentiate binding to the CD33 receptor from binding to the MCAT-1 receptor. The rationale for the TR constructs will be discussed below.

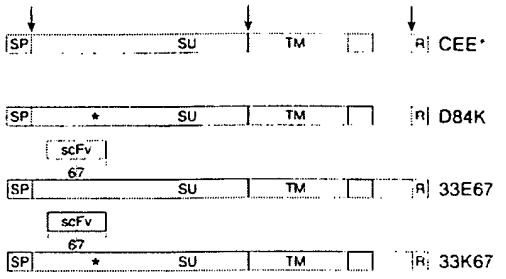

FIG. 1. Schematic diagrams of the chimeric envelope protein constructs. Mo-MuLV envelope expression vector CEE+ was engineered to contain a *Sfi*I site (5′ end) and a *Not*I site (3′ end) between amino acids 6 and 7, yielding the vector named E67. A CD33 scFv was amplified by PCR and the PCR product was subcloned into E67 at the *Sfi*I and *Not*I sites to obtain the chimeric envelope protein 33E67. A point mutant, D84K, that had been previously demonstrated to be a binding-defective mutant (17), was introduced into 33E67 to generate 33K67. Vertical arrows indicate proteinase cleavage sites. SP, signal peptide; SU, surface protein; TM, transmembrane protein; CEE+, wild-type ecotropic envelope protein. The approximate site of the binding mutant D84K is denoted by an asterisk. The membrane-spanning domain is denoted as a grey box. The other four constructs used in this study, CEETR, D84KTR, 33E67TR, and 33K67TR, are the R-peptide truncated forms of CEE+, D84K, 33E67, and 33K67, respectively.

Efficient Incorporation of Chimeric Envelope Proteins into Viral Particles. First it was important to demonstrate that the chimeric envelope proteins were translated, processed, and incorporated into viral particles as efficiently as the wild-type envelope protein. These data were obtained by Western blot analysis. Supernatants from cells transiently transfected with the envelope constructs were collected and centrifuged through 20% sucrose to pellet viral particles and eliminate free protein. These pellets were then analyzed on immunoblots for their gag (p30 CA) and envelope gp70 (SU) content. The amount of SU detected for each of the chimeric envelopes (Fig. 2, lanes 3, 4, 6, and 7) was similar to the amount of wild-type SU (Fig. 2, lanes 2 and 8). These data demonstrate that the chimeric envelope proteins can be expressed, processed, and incorporated into virions as efficiently as the wild-type envelope protein.

Efficient Binding of Viral Particles Carrying the CD33 Chimeric Envelope Protein to CD33-Expressing Cells. We next tested the efficiency with which the chimeric envelope proteins containing the CD33 scFv could bind to cells express-

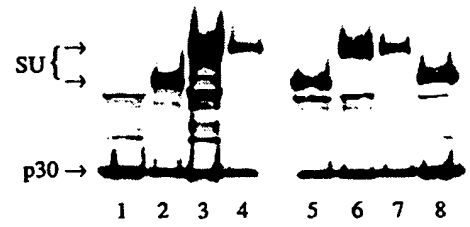

FIG. 2. Detection of envelope SU and p30 CA protein in virions by Western blot. The supernatants from transiently transfected cells were collected and centrifuged through 20% sucrose to pellet viral particles and remove free protein. Pellets were then analyzed on 8–16% SDS/PAGE gels for their gag (p30 CA) and envelope gp70 (SU) content. Viral SU could be detected for all the chimeric envelopes. SU containing the CD33 scFv migrates at a higher position than the wild type. Lanes: 1, mock transfection; 2, ecotropic viral particles from GPE86/LNCX producer cells (41); 3, 33E67; 4, 33E67TR; 5, D84K; 6, 33K67; 7, 33K67TR; 8, CEE+.

ing the CD33 receptor. We used a cell line, 3T3/CD33, which is 3T3 cells expressing CD33 (19). Viral particles containing each one of the chimeric envelope proteins were incubated with target cells, either NIH 3T3 or 3T3/CD33, and then analyzed by immunofluorescent flow cytometry to determine the efficiency of binding of each construct to the CD33 antigen or to the natural ecotropic MuLV receptor, MCAT-1 (Fig. 3). Viral particles with 33E67 (or 33E67TR, data not shown) efficiently bind to both 3T3 and 3T3/CD33 cells, while particles with 33K67 (or 33K67TR, data not shown) bind only to 3T3/CD33 cells, not to 3T3 cells. These data confirm that the envelope constructs containing the CD33 scFv can bind efficiently to the CD33 antigen and that the envelope constructs containing the D84K binding mutation cannot bind to the MCAT-1 receptor. Furthermore, the binding of 33E67 to MCAT-1 indicates that the insertion of the CD33 scFv has not produced significant steric hindrance for binding by the chimeric envelope protein. Efficient binding of viral particles carrying the CD33 chimeric envelope protein could also be demonstrated on HL60 and NB4, which are human cells from myeloid lymphoma patients that carry the CD33 receptor but not the MCAT-1 receptor (data not shown). Thus, the chimeric envelope proteins were processed and incorporated efficiently and could specifically bind to the CD33 antigen on target cells.

Viral Particles Carrying CD33 Chimeric Envelope Proteins Cannot Transduce Cells. Because HL60 and NB4 cells cannot be transduced with ecotropic retrovirus and only inefficiently with wild-type amphotropic virus (data not shown), we chose NIH 3T3 and 3T3/CD33 cells as a model system to study the transduction by the chimeric viral particles. We could not detect titer on either cell line with the supernatant from 33E67, 33E67TR, 33K67, or 33K67TR, although the control, CEE+, produced the expected titer of 4×10^6 on both cell lines (data not shown). Thus, despite the fact that the binding of 33E67 with both cell lines is as strong as wild type, no transduction of the target cells took place. The barrier occurs not only when the interaction of the chimeric envelope is with the CD33 receptor (i.e., with 33K67, which cannot bind MCAT-1 on 3T3/CD33 cells), but also when binding is occurring with the native receptor (i.e., with 33E67 on 3T3 cells). Therefore, a block occurs at some stage after binding of the viral particle to receptor that results in the inability of the vector genome to be expressed in the target cell.

No Reverse Transcription Occurs with Viral Particles Carrying CD33 Chimeric Envelope Proteins. To locate the postbinding block that prevents target cell transduction, we first asked whether the viral particles that bound to the CD33 receptor could release their cores into the target cell cytoplasm. If so, then it should be possible to detect preintegration complexes in the cytoplasm. After core entry, reverse transcriptase in the viral core results in the conversion of the single-stranded RNA viral genome into linear double-stranded DNA. This vector DNA can be detected by Southern analysis. NIH 3T3 or 3T3/CD33 cells were incubated with viral particles for 6 hr and then the cytoplasmic DNA was isolated. A ^{32}P-dCTP-labeled long terminal repeat

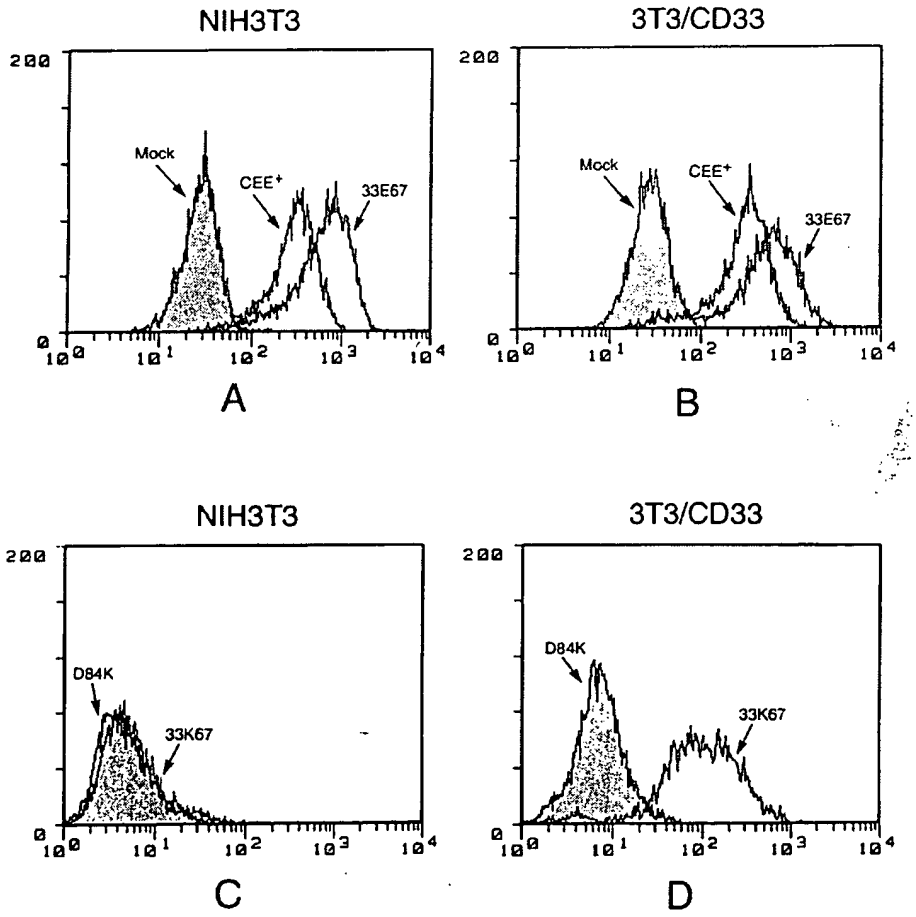

FIG. 3. The strength of envelope/receptor binding of the retroviral envelope constructs. Supernatants containing viral particles from transient transfections were collected and applied to target cells, either NIH 3T3 or 3T3/CD33 cells, to detect the binding of chimeric envelope to the CD33 antigen. Binding of viral particles to the target cells was detected by an immunofluorescent flow cytometry assay. The binding signals of control incubations (using either D84K or mock transfection supernatant) are shown as grey control peaks.

fragment from the retroviral vector was used as a probe to detect retroviral DNA (Fig. 4). In both NIH 3T3 and 3T3/CD33 cells, retroviral DNA signals were detected only for wild-type (CEE+) vector particles (Fig. 3, lanes 3 and 9). No signal was detected for any of the other constructs, including 33E67, which binds to the same receptor, MCAT-1, equally as efficiently as wild-type viral particles. These data indicate that even though the chimeric viruses could bind to the target cells, reverse transcription of viral sequences did not occur.

Viral Particles Carrying CD33 Chimeric Envelope Proteins Can Be Internalized. Because we could not detect reverse-transcribed viral sequences despite chimeric envelope binding to the target cell, the postbinding block may be the consequence of blockage before or at core release or later at the reverse-transcription step itself. We asked whether the chimeric viral particles could at least be internalized after binding to CD33 or to MCAT-1. Internalization is defined as the process whereby a molecule or macromolecular complex moves from the extracellular side of the cell membrane into the cytoplasm. There are a number of mechanisms whereby this process can occur (27). The one assumed to be active for Mo-MuLV entry is receptor-mediated endocytosis (28). With certain cell lines like 3T3, SC-1, and Rat-1 cells, retroviral infection is sensitive to lysosomotropic agents (i.e., inhibitors that buffer lysosomal pH such as NH_4Cl and chloroquine), suggesting that either virus–cell membrane fusion or core entry needs to be in a low pH environment (28, 29). We used immunoprecipitation and EM to investigate internalization.

Viral particles carrying ^{35}S-labeled chimeric envelope protein were bound to target cells by incubating for 2 hr at 4°C followed by extensive washing and then incubation for 1 hr at 37°C to allow for internalization if it could occur. After trypsinization to remove cell surface-bound virions, the cell lysates were immunoprecipitated with anti-SU and anti-p30 antibodies and then electrophoresed on a PAGE gel (Fig. 5). Bands for both 33E67 and 33K67 chimeric envelope protein and p30 protein could be detected in 3T3/CD33 cells (Fig. 5, lanes 1 and 2); the p30 protein could also be detected but at a much lower level with viral particles that carried no envelope protein. As a control, samples treated with trypsin after binding were used to examine the background signal coming from particles that trypsin failed to remove. The signals from these samples were significantly weaker than those from the experimental samples (data not shown). Therefore, by immunoprecipitation, internalization of the viral particles appears to take place.

EM was used to observe the presence or absence of retroviral particles inside the cell membrane. After 1-hr incubation of viral supernatants with 3T3/CD33 cells, viral particles carrying either wild-type envelope (CEE+) or the chimera 33E67 or 33K67 were found in significant numbers inside the cell membrane, while viral particles carrying the mutant envelope protein D84K were present in lesser amounts (Table 1). The difference between particles containing 33E67 or

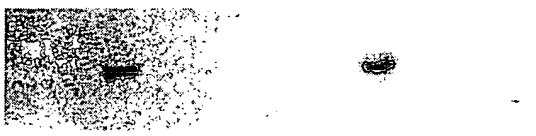

FIG. 4. Detection of preintegration complex in target cells. Supernatants containing viral particles from transient transfections were incubated with NIH 3T3 or 3T3/CD33 cells for 6 hr at 37°C. The cytoplasmic DNA was isolated and analyzed by Southern blot. The probe used in the study was a ^{32}P-dCTP-labeled long terminal repeat fragment (24). Lanes: 1 and 7, mock transfection (with the pHIT60 and pCnB plasmids, but no envelope protein plasmids); 2 and 8, D84K; 3 and 9, CEE+; 4 and 10, 33E67; 5 and 11, 33K67; 6 and 12, H_2O transfection. Lanes 1–6 are from 3T3 cells and lanes 7–12 are from 3T3/CD33 cells.

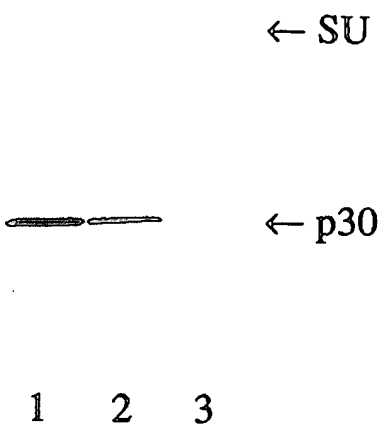

FIG. 5. Internalization of bound viral particles. 3T3/CD33 cells were used as the target cells. After incubating target cells at 4°C with viral particles for 2 hr, cells were washed with PBS and incubated in D10 medium for 1 hr at 37°C, followed by washing three times with PBS and then incubation in trypsin/EDTA for 10 min at 37°C. Lysates of cells were analyzed by immunoprecipitation with anti-SU and anti-p30 antiserums. Lanes: 1, 33K67; 2, 33E67; 3, mock transfection (with pHIT60 and pCnB plasmids, but no envelope protein plasmids).

33K67 with CEE+ is not statistically significant ($P > 0.05$), but the P value between particles containing D84K vs. CEE+ is significantly different ($P < 0.01$). Likewise, the P value is significant (<0.01) when particles carrying 33K67 are incubated with 3T3 cells, which do not have a receptor for CD33. These data demonstrate that retroviral particles are mainly internalized by receptor-mediated endocytosis, although some nonreceptor-mediated internalization occurs. Thus, data from both immunoprecipitation and from EM suggest that viral particles that can bind to a receptor can also be internalized.

However, a caveat is that these analyses are complicated by the fact that there are a large number of noninfectious virus-like particles in every virus preparation. It is not possible to distinguish defective from nondefective particles by either immunoprecipitation or by EM. Therefore, it is an assumption that the nondefective particles have properties similar to the bulk of the particles in the virus preparations studied.

Viral Particles Carrying CD33 Chimeric Envelope Proteins Cannot Carry out Fusion. The fusion of viral and cellular membranes is necessary for enveloped virus entry. Fusion occurs either in the endosome, where the low pH is thought to trigger a conformational change in the envelope protein to release the fusion peptide such as occurs with the influenza envelope protein HA1 (30–32), or on the cell membrane such as occurs with many retroviruses, including HIV (33). For

Table 1. Electron microscopy study of viral particle internalization

Envelope protein	n	Cell line	Particles per cell		P value
			Mean*	Range†	
CEE+	12	3T3/CD33	4.7 ± 1.7	1–23	—
D84K	24	3T3/CD33	0.9 ± 0.2	0–3	<0.01
33E67	24	3T3/CD33	3.4 ± 0.6	0–11	>0.05
33K67	24	3T3/CD33	3.4 ± 0.4	1–11	>0.05
33K67	12	3T3	1.0 ± 0.03	0–3	<0.01

Chimeric and wild-type viral particles were incubated with 3T3/CD33 or with 3T3 cells for 1 hr at 37°C. The cells were then trypsinized and fixed for EM study, and the number of viral particles inside the cell membrane was counted. n, number of cells analyzed; *, mean ± SE; †, minimum and maximum number of viral particles found in any single cell. The P value is for the comparison between the indicated chimera (or mutant) vs. wild type.

Table 2. Titer and fusion ability of viral particles containing heterooligomers composed of two separate chimeric envelope proteins

DNA	CEETR	D84KTR	33E67TR	33K67TR
CEE+	+(7.0×10^6)	+(6.8×10^6)	+(3.5×10^6)	+(3.0×10^6)
D84K	+(5.3 ± 10^6)	−(0)	−(0)	−(0)
33E67	+(1.7×10^6)	−(0)	−(0)	−(0)
33K67	+(1.4×10^6)	−(0)	−(0)	−(0)

To determine titer, supernatants from transfected cells were collected and added to 3T3/CD33 cells. After 10 days of selection in G418 (0.6 mg/ml), G418-resistant colonies were counted (data in parentheses). Except for CEE + (4×10^6) and CEETR (2.5×10^2), the titer of the supernatant from each of the other single DNA transfections is zero. Syncytia formation of 3T3/CD33 cells mediated by transfected envelope protein plasmid is indicated by a "+," and no syncytia, by a "−." Unless monomers of the wild-type (CEE+) or R-less wild-type (CEETR) envelope protein are present in the mixed heterooligomer, no syncytia formation occurs (see text).

Mo-MuLV, a low pH step seems to be required for virus entry, because infection of certain cell lines is sensitive to lysosomotropic agents (28, 29, 34). However, in XC cells, expression of retroviral envelope protein alone can mediate cell–cell fusion as demonstrated by syncytia formation, and this syncytia assay has been widely used to study the fusogenicity of the ecotropic murine retroviral envelope protein (35). The expression of chimera 33E67 in XC cells does not elicit syncytia formation (data not shown), thereby suggesting that the postbinding block is at the step of virus–cell membrane fusion.

Viral Particles Carrying CD33 Chimeric Envelope Proteins Cannot Carry out the Steps Required for Fusion. Because the XC fusion assay does not provide information on the mechanism that produces the block in fusion, we used an assay that our laboratory has recently developed (22, 36) to examine this critical step.

The rationale for this assay is as follows. The Mo-MuLV envelope protein contains two subunits, SU and transmembrane protein (TM). The final 16 residues of the TM (R-peptide) strongly influences envelope fusogenicity (18, 25, 26). As noted above, it has been shown that Mo-MuLV envelope protein expression will allow syncytia formation of XC cells, but this does not occur with NIH 3T3 cells unless the fusogenicity of the envelope protein is enhanced. It was demonstrated (25, 26) that the truncated R-peptide (R-less) ecotropic envelope protein (i.e., CEETR) can induce massive syncytia formation of 3T3 cells, while the wild-type envelope (CEE+) cannot.

On the virion, the envelope protein forms an oligomer, most likely a trimer (37, 38). We have recently demonstrated that there is a functional interaction among the monomers in this trimeric structure (22, 36), and supporting evidence for this hypothesis has recently been published by others (39). For example, when D84KTR (a binding-defective mutant in the R-less form that cannot induce syncytia formation of 3T3 cells because it cannot bind) is coexpressed with CEE+ (which cannot induce syncytia because it is not R-less), massive syncytia formation occurs. This phenomenon was shown to result from a functional interaction within the coexpressed heterotrimer, which allows different monomers to complement each other functionally. Specifically in this example, the required conformational change of the monomer from CEE+ that occurs after its binding with its receptor complements the monomer D84KTR, which cannot bind and therefore cannot initiate its own conformational change, but can undergo the conformational change when triggered by the CEE+ monomer in the heterotrimer. D84KTR can then contribute the R-less phenotype for syncytia formation of 3T3 cells. Thus, this functional interaction assay has been demonstrated to assay functionally, although not structurally, for the conformational change that occurs in the Mo-MuLV envelope protein during the fusion step.

To analyze the postbinding block in our chimeric vectors, we applied this assay to determine whether the CD33 chimeric envelope protein could successfully interact with an adjacent monomer in a trimer after it binds to its CD33 receptor. The entire matrix of interactions was measured between the four R-less constructs and their four R-containing counterparts: CEETR, D84KTR, 33E67TR, or 33K67TR complemented individually with CEE+, D84K, 33E67, or 33K67 (see Table 2 and Fig. 6). *If* the CD33 chimeric envelope proteins can undergo the fusion-required conformational change, then functional interaction among the monomers within a heterotrimer will allow syncytia formation with certain combinations. The data are clear. No syncytia are observed in any of the relevant combinations of chimeric envelopes. Thus, there are no instances of a functional interaction, and therefore it would appear that there has not been a correct conformational change in the chimeric envelope protein.

An important result was obtained with 33E67. Because the putative conformational change is blocked even with binding to the natural MCAT-1 receptor (33E67 plus D84KTR, 33E67TR, or 33K67TR are zero, as are D84K or 33K67 plus 33E67TR), this result suggests that the presence of the CD33 scFv sufficiently disrupts the normal architecture of the envelope protein so that the normal conformational change that

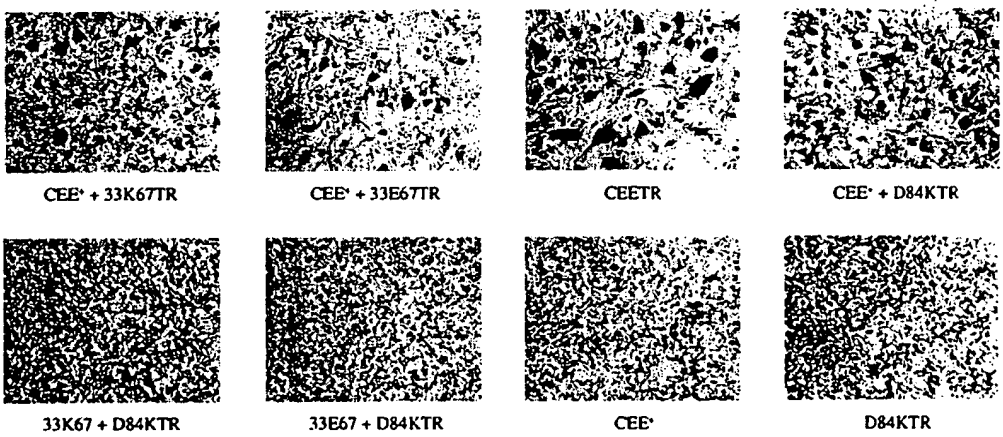

FIG. 6. Chimeric envelope protein-mediated syncytia formation of 3T3/CD33 cells. Envelope protein expression plasmids were cotransfected (at a ratio of 1:1) into 3T3/CD33 cells. At 36 hr after transfection, the cells were stained with methylene blue, and those cells containing more than four nuclei counted as syncytia. (*Upper*) Examples of positive syncytia formation; (*Lower*) Examples of negative syncytia formation. These data are a portion of those summarized in Table 2.

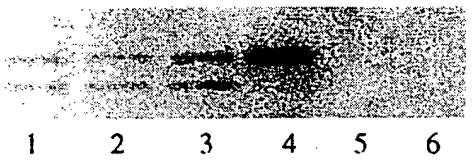

FIG. 7. Heterooligomer formation between chimeric envelope monomers and wild-type monomers. SDS/PAGE (14%) of the coimmunoprecipitated virion envelope protein is shown. Envelope proteins were transiently expressed in 293T cells. Metabolic labeling for 4 hr with [^{35}S]Met was performed 24 hr after transfection. The cells were lysed, and the supernatant was immunoprecipitated with 5 μ of anti-R peptide (22). The p12E protein, bottom band, that was present in 33E67TR and 33K67TR, was immunoprecipitated by R-peptide antiserum only in the presence of coexpressed D84K (lane 1, 33E67TR/D84K; lane 2, 33K67TR/D84K); or CEE+ (lane 3, 33K67TR/CEE+), but not when expressed by itself (lane 5, 33E67TR and lane 6, 33K67TR). There is no p12E band in the wild-type CEE+ (lane 4), indicating that there is no R-peptide cleavage in this 293T system.

would occur after MCAT-1 receptor binding cannot take place. Thus, the putative conformational change cannot occur whether the binding is to the CD33 receptor or to the MCAT-1 receptor on the target cell.

An alternative explanation for a lack of a functional interaction could be that the protein architecture of the envelope protein was sufficiently disrupted by the anti-CD33 scFv that heterooligomer formation was prevented. If this were the case, a failure to observe a functional interaction could not be attributed to a loss of a functional conformation change. To determine whether heterooligomer were indeed formed between the chimeric and the D84K envelope protein, coimmunoprecipitation assays were performed. We took advantage of the fact that Mo-MuLV envelope protein expressed in the absence of the viral protease will retain the R-peptide. Antibody to R-peptide can immunoprecipitate the R-less form of TM (p12E), in addition to the full length form of TM (p15E), when both full length and R-less forms of the wild-type envelope are coexpressed in the same cell (22). 293T cells lack viral proteinase activity so that the R-peptide will not be cleaved from the full length protein in these cells. The presence of p12E in the precipitate is evidence for an interaction between p15E and p12E molecules.

Coimmunoprecipitation was performed to test each of the relevant combinations for the presence of heterooligomer. With R-peptide anti-serum, p12E bands could be precipitated only when 33E67TR or 33K67TR was coexpressed with D84K or CEE+ (Fig. 7, lanes 1–3), but not when they are expressed alone (Fig. 7, lanes 5 and 6). Thus, heterooligomer are formed between chimeric and wild-type envelope protein monomers.

Conclusion. The conclusion from these data is that the binding of the chimeric envelope protein with receptor on the target cell does not, in itself, lead to successful fusion of the viral and cellular membranes, a prerequisite for the transfer of the viral core into the target cell. Although our data cannot rigorously establish a precise mechanism, the most likely explanation is that the chimeric envelope is not able to undergo the conformational change that triggers the fusion process. Whether the binding is to the native receptor (MCAT-1) or to the targeted molecule (CD33), this triggering process does not occur with the chimeric envelope proteins.

Even though the mechanism causing the postbinding block that we have demonstrated in our study may not be true for every chimeric retroviral envelope protein, it is reasonable to assume that it may be a major cause for the poor transduction efficiency seen in many chimeric retroviral systems. Before efficient targeted transduction by using retroviral vectors can be achieved, it will probably be necessary to develop a much better understanding of the structure/function of the wild-type envelope protein itself. Recent x-ray crystallographic studies of the ecotropic envelope protein (38, 40), although providing only structural information on parts of the envelope protein, are valuable contributions to this understanding (B. Wu and W.F.A., unpublished work). Vectors that could efficiently transduce specific cell types *in vivo* would be invaluable tools for clinical gene therapy protocols.

We thank Guoliang Li for excellent technical assistance, Ernesto Barron from the University of Southern California Electron Microscopy Core Facility for his assistance with electron microscopy, and Paula Cannon, Nori Kasahara, and Cyril Empig for helpful discussions and for critical reading of the manuscript. We also thank Chris Benedict for supplying the E67 plasmid. This work was supported by Genetic Therapy, Inc. (GTI)/Novartis and by National Institutes of Health grant CA59318.

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Exhibit A

Virology 269, 7–17 (2000)
doi:10.1006/viro.2000.0201, available online at http://www.idealibrary.com on IDEAL®

Identification of Regions in the Moloney Murine Leukemia Virus SU Protein That Tolerate the Insertion of an Integrin-Binding Peptide

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Received September 28, 1999; returned to author for revision December 10, 1999; accepted January 11, 2000

Targeting of retroviral vectors to specific cells has been attempted through engineering of the surface (SU) protein of the murine leukemia viruses (MuLVs), but in many cases this has adversely affected protein function and targeted delivery has been difficult to achieve. In this study, we have inserted a 15-mer peptide that binds specifically to the $\alpha_v\beta_3$ integrin into the Moloney MuLV SU protein, including regions that are surface exposed in the crystal structure of the ecotropic receptor-binding domain. We have concentrated in particular on the variable regions VRA, VRB, and VRC, which are responsible for the use of distinct cellular receptors by different MuLV subtypes and therefore may be more likely to accommodate a heterologous binding moiety. Despite these considerations, only 8 of 26 insertion sites were tolerated, including two separate regions in VRA, a cluster of sites in VRC, and previously identified sites at the N-terminus of the protein and in the proline-rich region immediately downstream of the receptor-binding domain. When expressed on retroviral vector particles, all of the viable proteins retained the ability to bind to and transduce murine cells, although the VRC mutants and an insertion in VRA gave reduced binding and titer. Finally, although all of the viable chimeras could bind to $\alpha_v\beta_3$ in a solid-phase binding assay, we were unable to demonstrate expanded tropism for $\alpha_v\beta_3$-expressing human cells. This study highlights the difficulty of engineering the Moloney MuLV SU protein, even when structural information is available, and provides guidelines for the insertion of peptide ligands into the SU protein. © 2000 Academic Press

INTRODUCTION

The ability of retroviruses to integrate into the host cell genome has led to the popularity of retroviral vectors in gene transfer protocols where the stable introduction of a therapeutic gene is required (Anderson, 1998). However, a major limitation to the clinical use of such vectors is the lack of specificity of delivery to defined target cells. Such targeted transduction would effectively increase the titer of the vectors by preventing the transduction of nontarget cells, and for toxic therapeutic gene products, such as those proposed for certain anti-tumor strategies, specific delivery would also minimize unwanted side effects. Previous attempts to achieve targeted transduction have concentrated on the manipulation of the envelope (Env) protein on the surface of the retroviral particle, which interacts with a specific host cell surface receptor and is the primary determinant of viral tropism (reviewed in Hunter, 1997).

The murine leukemia virus (MuLV) Env is believed to be a homotrimer (Kamps *et al.*, 1991). Each monomer is synthesized as a single-chain precursor, Pr85, that is later processed into two subunits, the surface (SU) protein and the transmembrane (TM) protein (Pinter and Honnen, 1983; Freed and Risser, 1987). The SU protein mediates the attachment of the viral particle to its host cell receptor, while the TM protein is required for the membrane fusion events that occur after receptor binding (White, 1992). The SU protein can be divided into three regions: a variable N-terminal receptor-binding domain (RBD) (Heard and Danos, 1991; Battini *et al.*, 1995, 1996; Davey *et al.*, 1999), a more conserved C-terminal domain that associates with the TM protein (Pinter and Fleissner, 1977; Hunter, 1997), and a hypervariable proline-rich region (PRR) that links the two (Koch *et al.*, 1983; Ott *et al.*, 1990; Wu *et al.*, 1998). Within the N-terminal RBD, amino acid sequence alignments have identified three variable regions, designated VRA, VRB, and VRC, which contain most of the sequence differences between the different MuLV subtypes and are primarily responsible for the specificity of receptor interaction (Battini *et al.*, 1992; Fass *et al.*, 1997).

Ecotropic MuLVs infect virtually all rodent cells but not human cells. In order to develop a targeted retroviral vector, a feasible approach is to expand the host range of an ecotropic MuLV vector by introducing an additional targeting moiety into Env (Cosset and Russell, 1996; Schnierle and Gromer, 1996). At present, a common strategy is the addition of a protein domain or single-chain antibody (scFv) to the SU protein (Russell *et al.*,

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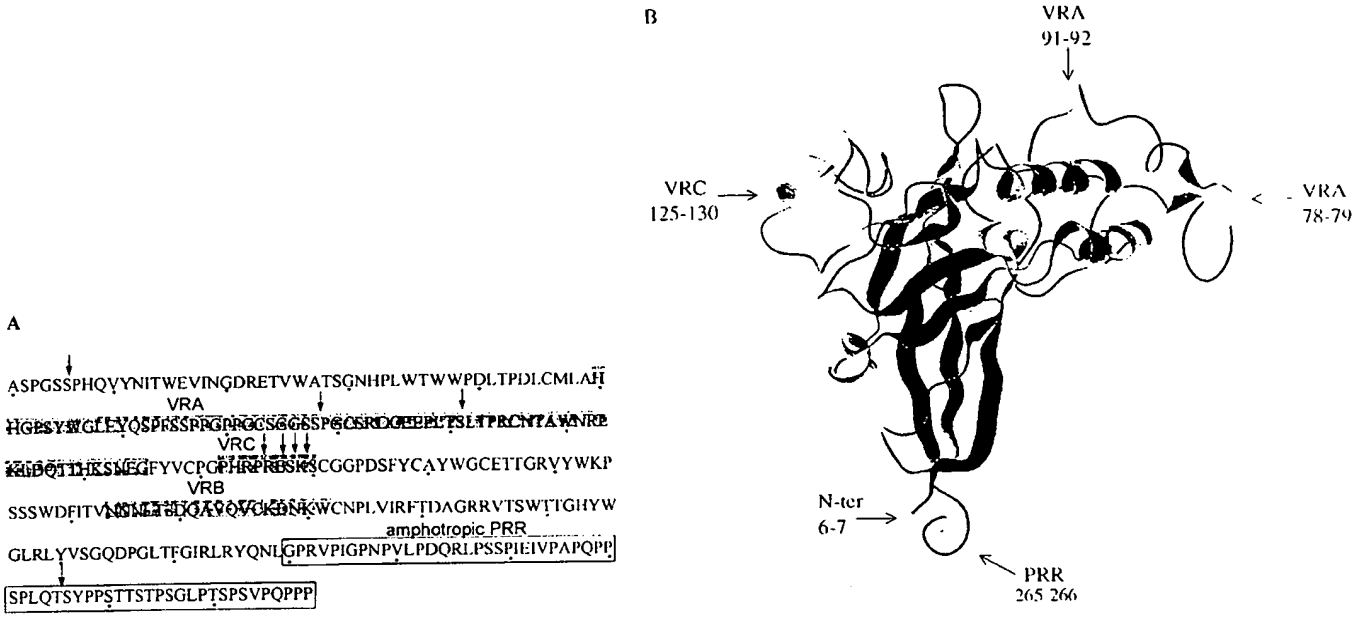

FIG. 1. Peptide insertions in the MoMuLV RBD. (A) Amino acid sequence of the N-terminus of the MoMuLV SU protein; VRA, VRB, and VRC as defined (Fass *et al.*, 1997) are shaded. The proline-rich region (PRR) is boxed and the sequence shown is derived from the amphotropic Env protein, as is present in plasmid E/A-PRR used as the backbone for the insertion in this region (Wu *et al.*, 1998). The locations of the peptide insertion sites that generated viable chimeric Env proteins are arrowed. (B) Location of viable insertion sites in MoMuLV Env indicated by the homologous positions in the crystal structure of the FrMuLV RBD (residues 9 to 236). Neither position 6–7 nor position 265–266 is included in the crystal structure, but their relative positions are estimated for illustrative purposes.

1993; Benedict *et al.*, 1999; Zhao *et al.*, 1999) or the replacement of much of the RBD with such a ligand (Kasahara *et al.*, 1994). However, such approaches can result in lower levels of the chimeric Env being incorporated into viral particles when compared to the wild-type protein and coexpression of the wild-type protein may be required. In addition, the fusion capacity of chimeric Env proteins is often dramatically impaired (Benedict *et al.*, 1999; Zhao *et al.*, 1999). As a result of these considerations, we chose to use a small peptide ligand as a targeting motif, expecting that this would be minimally disruptive to the overall structure of the SU protein and to processes such as transport to the cell surface, precursor processing, incorporation into particles, and fusion activity.

In this study, a 15-amino-acid peptide that binds specifically to the vitronectin receptor, $\alpha_v\beta_3$ (Healy *et al.*, 1995), was inserted into the SU domain of the Moloney MuLV (MoMuLV) Env, in particular at sites predicted to be surface-exposed loops in the three-dimensional structure of the monomer of the highly homologous Friend MuLV (FrMuLV) RBD (Fass *et al.*, 1997). Eight sites in the receptor-binding domain of the SU protein were identified that tolerated the insertion of the peptide, including the region between residues 6 and 7, two sites in VRA, a cluster of sites in VRC, and the PRR. In addition, certain combinations of double insertions were also tolerated. Our analysis of the binding of the chimeric Env proteins to $\alpha_v\beta_3$ in a solid-phase binding assay revealed differences in their abilities, which may reflect the effects of structural constraints from the surrounding Env protein scaffold on the accessibility and function of the targeting peptide. Finally, the properties of the chimeric proteins suggested that the VRC region may be important for binding of Env to the ecotropic receptor, in addition to the previously identified VRA region (MacKrell *et al.*, 1996; Davey *et al.*, 1999), as insertions in VRC reduced both binding to the ecotropic receptor and titer on murine NIH 3T3 cells.

RESULTS

Rationale for selection of peptide insertion sites. Previous attempts to insert binding moieties into the MoMuLV Env protein have frequently met with the problem that the chimeric Env proteins generated were not efficiently processed and incorporated into retroviral particles (Benedict *et al.*, 1999). In order to increase our chances of successfully inserting a targeting moiety into the SU protein, we chose to use a small peptide rather than a larger protein domain or scFv. The targeting peptide used was a linear 15-amino-acid RGD peptide that has previously been shown to preferentially bind to the vitronectin receptor, $\alpha_v\beta_3$ (Healy *et al.*, 1995). For the insertions in the SU protein, we selected sites predicted to be surface exposed by the crystal structure of the monomeric RBD of FrMuLV (Fass *et al.*, 1997) and therefore more likely to tolerate such insertions. In particular, we chose to concentrate on the variable regions VRA, VRB, and VRC (Fig. 1), as these regions naturally contain

sequence variation allowing the use of different receptors and therefore may be more tolerant of the peptide insertions. In addition, we also inserted the peptide into two regions outside the RBD structure that have previously been shown to tolerate the insertion of binding moieties: between amino acids 6 and 7 at the N-terminus of SU (Russell *et al.*, 1993; Benedict *et al.*, 1999; Zhao *et al.*, 1999) and in the hypervariable PRR (Wu *et al.*, 1998).

VRA (residues 50 to 115 in MoMuLV) consists of a helical region (residues 97–110) that allows a more extended loop region to project away from the β-sandwich body of the RBD (Fass *et al.*, 1997). Within this extended region there are two disulfide bonds that link cysteines 72 and 85, and 73 and 81, respectively (Linder *et al.*, 1992), and that give rise to two cysteine-constrained loops comprising residues 74–80 and 82–84. We have previously demonstrated that residues in the MoMuLV VRA are important for receptor binding, in particular residue D84 (MacKrell *et al.*, 1996). Analysis of the structure of the region surrounding the equivalent aspartic acid residue in the FrMuLV RBD has led to the suggestion of a receptor-binding face that includes a charged ridge (equivalent MoMuLV residues R83, E87, P88, and T90) and a hydrophobic pocket (residues P88, L89, W100, and L92) (Fass *et al.*, 1997). Accordingly, we made several insertions in this region that we predicted would place the RGD peptide at, or close to, the authentic receptor-binding site. We replaced residues 74–80 and 74–84 in the cysteine-constrained loops with the RGD peptide and also inserted the peptide at two positions within these loops, between amino acids 78 and 79 and amino acids 83 and 84. In addition, we made an insertion between amino acids 91 and 92, close to the hydrophobic pocket. Finally, we also inserted the peptide between residues 54 and 55, 59 and 60, and 69 and 70, which are more distant from the putative receptor contact face but are located in highly variable portions of the extended VRA region, and also at sites 93–94 and 97–98, which are located close to the VRA helix.

We also made several insertions at surface-exposed sites in the VRC region (residues 120 to 130) and the VRB region (residues 167 to 169). VRB has also been implicated as a receptor-binding determinant for the MuLV subtypes (Battini *et al.*, 1995; Han *et al.*, 1997), while VRC has been suggested to be part of the putative ecotropic receptor-binding site by virtue of lying at the interface of monomers in a computer-generated model of a trimer of the FrMuLV RBD (Fass *et al.*, 1997). Finally, we also inserted the peptide into several regions that are conserved between the different MuLV subtypes, but are surface exposed in the RBD crystal structure.

Identification of sites that tolerate peptide insertions. Twenty-six Env proteins containing peptide insertions were generated and the chimeric proteins were assessed for their ability to be processed and incorporated into retroviral vectors (Table 1, Fig. 1). The majority of the peptide insertions did not result in a functional Env protein that could be detected in retroviral particles and analysis of cell lysates revealed that they were defective in protein processing of the Env precursor, Pr85, to the mature SU protein, gp70, indicating a block in the transport pathway. However, several sites were identified that did tolerate the peptide insertion, including site 6–7 at the N-terminus of SU, sites 78–79 and 91–92 in the extended coil of VRA, sites 125–126, 127–128, 128–129, and 129–130 in the more exposed part of VRC, and position 265–266 in the PRR.

TABLE 1

Peptide Insertions in MoMuLV SU

Insertion site[a]	Location in SU[b]	Processing[c]	Incorporation[d]
6–7	N-terminus	+++	+
54–55	VRA	–	–
59–60	VRA	–	–
69–70	VRA	–	–
74–80	VRA	–	–
74–84	VRA	–	–
78–79	VRA	++	+
83–84	VRA	–	–
91–92	VRA	+	+
93–94	VRA	–	–
97–98	VRA	–	–
112–113	VRA	–	–
121–122	VRC	–	–
123–124	VRC	–	–
125–126	VRC	+	+
127–128	VRC	+	+
128–129	VRC	++	+
129–130	VRC	++	+
141–142	Conserved	–	–
150–151	Conserved	–	–
167–168	VRB	–	
168–169	VRB		
210–216	Conserved		
212–213	Conserved		
213–214	Conserved		
265–266	PRR	+++	+

[a] RGD peptide was inserted between amino acids (e.g., 6–7) or used to replace amino acids (e.g., 74–84) of MoMuLV Env in plasmid pCEE+; insertion 256–266 was in plasmid E/A-PRR.

[b] VRA, VRB, and VRC regions vary in sequence between different MuLV subtypes, while the N-terminus and the conserved regions do not; PRR is the proline-rich region.

[c] Extent of Pr85 to SU protein processing in lysates of 293T cells at 37°C. +++, wild-type level of processing with more SU protein than Pr85; ++, less SU protein than Pr85; +, low level of processing; –, only Pr85 detected.

[d] SU protein present (+) or not (–) in retroviral vector particles generated at 37°C.

Peptide insertions cause temperature-sensitive defects in protein processing. We analyzed in more detail the form of the Env proteins present in cell lysates and retroviral particles for the panel of viable chimeras. 293T

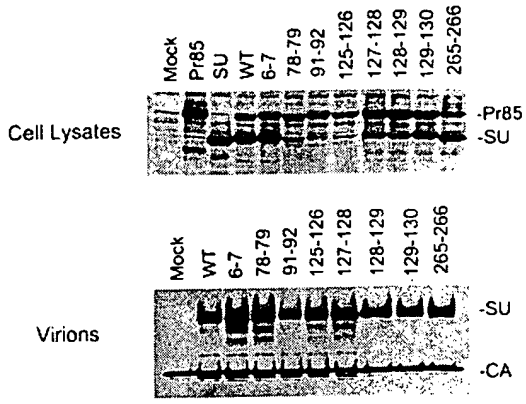

FIG. 2. Western blot analysis of chimeric Env proteins produced at 37°C. (A) Cell lysates. Env proteins were expressed in 293T cells and cell lysates were deglycosylated and probed with an anti-SU antibody. Pr85 is the precursor form and SU is the mature processed form of Env. Construct Pr85 contains a mutated SU–TM cleavage site and therefore produces only Pr85, while construct SU is an Env protein prematurely truncated at the SU-TM cleavage site and therefore expresses only SU. The WT construct expresses the wild-type MoMuLV Env protein and "Mock" is a transfection without any Env expression plasmid. (B) Virions. Viral particles were harvested from the supernatant of 293T cells and partially purified by pelleting through 20% sucrose. The SU and capsid (CA) proteins were detected with appropriate antibodies.

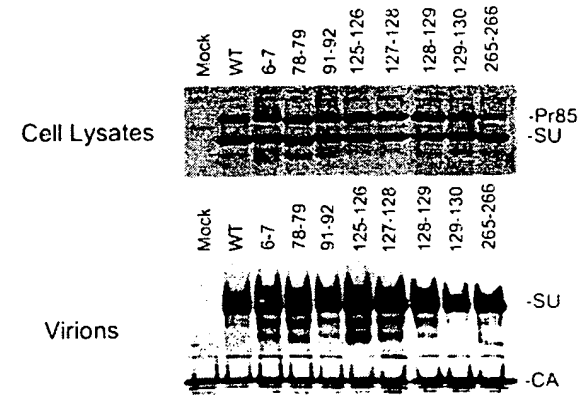

FIG. 3. Western blot analysis of Env proteins produced at 32°C. Proteins were analyzed as described in the legend to Fig. 2. (A) Cell lysates. All the chimeric proteins were processed efficiently from the precursor form, Pr85, to the mature form, SU. (B) Virions.

cells were transfected with Env protein expression plasmids and Western blot analysis was performed on deglycosylated cell lysates in order to obtain a clearer separation of Pr85, the SU protein, and possible processing intermediates (Fig. 2A). In addition, retroviral particles containing the chimeric Env proteins were partially purified through 20% sucrose and analyzed for the presence of the SU protein (Fig. 2B).

The results revealed that the peptide insertions caused varying effects on the efficiency of processing of Pr85 to SU protein. Insertions at 6–7 and 265–266 resulted in almost wild-type levels of Env processing, while insertions at 78–79, 127–128, 128–129, and 129–130 caused a reduction in the efficiency of processing. The insertions at sites 91–92 and 125–126 produced the most seriously affected chimeras, as only trace amounts of the mature form of the SU protein could be detected in the cell lysates. In contrast to the results from the cell lysates, the incorporation of chimeric Env proteins into viral particles was efficient for all of the chimeras, with the majority of the proteins being incorporated into virions at levels similar to the wild-type Env. Even for the peptide insertions that resulted in poor protein processing in the cell lysates, significant amounts of the SU protein were still detected in the viral particles, although the 91–92 insertion site resulted in the lowest levels of SU protein detected.

Defects in protein processing, as observed for several of the chimeric Env proteins, can reflect alterations in a protein's overall structure and folding pathway and such defects are often less severe at lower temperatures. Accordingly, we repeated the analysis of the Env proteins at 32°C and observed that the processing of the chimeras and their subsequent incorporation into particles were improved (Fig. 3). In particular, for the 91–92 chimera, which displayed the most severe reduction in Env processing at 37°C, the shift to the lower temperature allowed near wild-type levels of processing and subsequent Env incorporation.

Interaction of chimeric Env proteins with the ecotropic receptor. We investigated whether the chimeric Env proteins still retained the capacity to interact with the ecotropic receptor. Retroviral vectors containing the chimeric proteins were produced at 37°C and titered on NIH 3T3 cells, which express the ecotropic receptor. The results (Table 2) revealed that the insertions at 6–7, 78–79, and 265–266 resulted in wild-type titers on NIH 3T3 cells, the insertions in VRC reduced titer by about

TABLE 2

Characterization of Chimeric Env Proteins Containing the RGD Peptide

Env protein	Viral titer (CFU/ml)[a]	Binding to NIH3T3 cells[b]
Wild-type	$(1.3 \pm 0.3) \times 10^5$	+++
6–7	$(1.8 \pm 0.9) \times 10^5$	+++
78–79	$(1.5 \pm 0.5) \times 10^5$	+++
91–92	$(3.4 \pm 2.3) \times 10^3$	−
125–126	$(1.4 \pm 1.6) \times 10^3$	±
127–128	$(2.4 \pm 2.7) \times 10^3$	−
128–129	$(2.2 \pm 0.2) \times 10^3$	±
129–130	$(2.5 \pm 1.1) \times 10^3$	±
265–266	$(2.0 \pm 0.1) \times 10^5$	+++

[a] Titer on NIH 3T3 cells of retroviral vectors pseudotyped by chimeric Env proteins, produced at 37°C. Each titer value is the average of three independent experiments ± standard error.

[b] Binding of retroviral particles pseudotyped by the chimeric Env proteins produced at 32°C to NIH 3T3 cells, as determined by FACScan analysis. +++, wild-type level of binding; ±, only a trace amount of binding was detected.

one order of magnitude, and the insertion at 91–92 resulted in titers that were three orders of magnitude lower than that of the wild-type.

To further analyze the ability of the chimeric Env proteins to interact with the ecotropic receptor, we performed an indirect immunofluorescence assay followed by flow cytometry analysis in order to measure the ability of the chimeric Env proteins to bind to NIH 3T3 cells (Table 2). The viral particles used in the binding assay were harvested at 32°C so that they would all contain similar amounts of the chimeric Env proteins (Fig. 3). The results were consistent with the titer data and revealed that the Env proteins with insertions at sites 6–7, 78–79, and 265–266 bound to NIH 3T3 cells at wild-type levels, while the insertions at site 91–92 in VRA and at the cluster of sites in the VRC region resulted in reduced abilities to bind to the ecotropic receptor.

Accessibility of inserted peptides to an anti-RGD peptide antibody. Although the peptide insertion sites we had chosen were predicted to be surface exposed in the monomeric RBD structure, their locations in an oligomeric Env protein complex may not be as accessible. Accordingly, we wished to determine the extent to which the peptides in the viable chimeras could be recognized by an anti-peptide antiserum. The chimeric Env proteins were expressed in 293T cells and the overall level of cell surface Env was examined by flow cytometry using an anti-SU monoclonal antibody, 83A25 (Evans *et al.*, 1990). The same cells were then examined for the ability of the Env proteins to be recognized by the anti-peptide antiserum (Fig. 4).

Among the chimeric envelope proteins, 6–7, 78–79, and 265–266 had wild-type levels of cell surface expression, as detected by the anti-SU antibody. However, only 78–79 and 265–266 were also recognized by the anti-RGD peptide antibody, indicating good exposure of the peptide ligand in these chimeras. The insertion at site 91–92 in VRA and all of the insertions in VRC resulted in reduced recognition by the anti-SU antibody, with the 129–130 chimera being particularly affected. Given the lower levels of Env processing detected in the cell lysates of these chimeric proteins (Fig. 2), it is highly likely that the lower levels of Env recognition by 83A25 reflected smaller amounts of Env proteins being expressed on the cell surface. Although we cannot rule out the possibility that lower levels of Env were detected due to interference with the antibody recognition site by the inserted peptide, we think that this is unlikely given that 83A25 recognizes an epitope downstream of the RBD domain of the SU protein (Evans *et al.*, 1990). Consequently, for this group of chimeric proteins, we are not able to conclude whether their lack of recognition by the antiserum resulted from lower overall levels of cell surface expression or whether the peptides inserted at these locations were not readily available to the antibody.

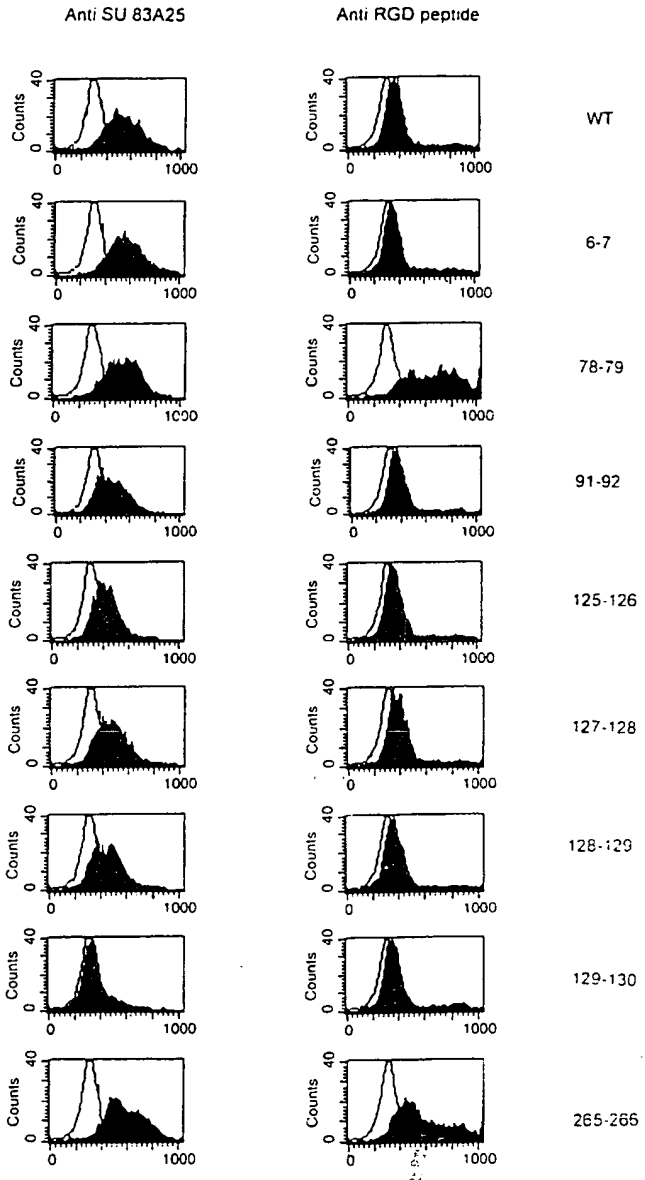

FIG. 4. Recognition of Env proteins by anti-SU and anti-RGD peptide antibodies. 293T cells were transfected by Env expression plasmids and surface Env proteins were detected using FACS analysis. The unshaded peaks represent cells that were stained with the secondary antibodies only and the shaded peaks represent cells that were stained with either the anti-SU antibody 83A25 (left) or the anti-RGD peptide antiserum (right).

Measurement of the binding of chimeric Env proteins to $\alpha_v\beta_3$. We used a solid-phase binding assay to measure the binding of the chimeric Env proteins to purified integrin $\alpha_v\beta_3$. Retroviral vector particles were harvested at 32°C to ensure good incorporation of each chimera. In addition, the particles were harvested in serum-free medium to minimize the amount of vitronectin in the viral sample. Vitronectin is a natural ligand of $\alpha_v\beta_3$ (Felding-Habermann and Cheresh, 1993) and is present at high levels in serum-containing medium.

The results of these analyses (Table 3) revealed that

TABLE 3

Binding of Chimeric Env Proteins to Integrin $\alpha_v\beta_3$

Envelope protein	Binding to $\alpha_v\beta_3$ receptor[a]
Mock	0.016 ± 0.009
Wild-type	0.036 ± 0.001
6–7	0.106 ± 0.013
78–79	0.268 ± 0.018
91–92	0.124 ± 0.008
129–130	0.152 ± 0.008
265–266	0.552 ± 0.027

[a] Binding capacity of the chimeric Env proteins to integrin $\alpha_v\beta_3$ was estimated by a solid-phase binding assay; the numbers represent the optical density at 490 nm, measuring the amount of Env protein remaining associated with the $\alpha_v\beta_3$-coated plate. Each value is the mean of two independent experiments.

insertion of the peptide at site 265–266 in the proline-rich region resulted in the highest binding affinity, while insertion at site 78–79 also resulted in a significant level of binding. In contrast, insertions at positions 6–7, 91–92, and 129–130 caused only low levels of binding to the integrin. These results were in good agreement with the data from the flow cytometry analyses that measured the accessibility of the peptides to the anti-peptide antiserum (Fig. 4). Taken together, these data suggest that peptides inserted at positions 78–79 in VRA and at 265–266 in PRR are highly accessible on the surface of Env and can bind to $\alpha_v\beta_3$. In contrast, the remaining insertion sites do not allow the peptide to interact well with its receptor. This could be due to poor exposure on the surface of the Env protein complex, constraints imposed by the surrounding protein sequences, or a combination of both factors.

Interaction of peptide-containing Env proteins with human cells. We examined the ability of the chimeric Env proteins to bind to a human melanoma cell line, M21, that expresses $\alpha_v\beta_3$ (Cheresh and Spiro, 1987) using indirect immunofluorescence followed by flow cytometry analysis. Except for the insertion at 265–266 that generated a small mean-channel shift, the rest of the proteins did not give a detectable binding signal (data not shown). We further examined whether retroviral vectors expressing the chimeric envelope proteins could transduce either M21 or MG-63 cells. Although vectors containing the amphotropic MuLV envelope protein gave titers of 4–6 × 10^4 colony-forming units (CFU)/ml on these cells, no detectable titer was observed for any of the viable chimeric Env proteins.

Simultaneous peptide insertions in two locations of SU protein. A short peptide ligand usually has only a modest affinity for its receptor, and this can be further reduced if the peptide is inserted into a heterologous protein. We therefore investigated the possibility of increasing the affinity of the chimeric Env proteins for $\alpha_v\beta_3$ by inserting the peptide simultaneously into two locations in the SU protein. Various combinations of insertions in the sites previously identified as viable were constructed and the resulting Env proteins were assessed by Western analysis of retroviral particles and by examining their ability to transduce NIH 3T3 cells (Fig. 5). Just as the single insertions at positions 6–7, 78–79, and 265–266 had no effect on viral titer (Table 2), we also found that the various combinations of these three insertion sites gave rise to fully functional Env proteins. The single insertion of a peptide at position 129–130 had previously reduced the titer on NIH 3T3 cells by one order of magnitude (Table 2) and the combination of 129–130 with any of these three innocuous insertion sites also resulted in chimeric proteins that gave similarly reduced titers. Overall, we found that there was no strict correlation between the efficiency of incorporation of the chimeric Env proteins into viral particles and subsequent titer on NIH 3T3 cells.

We also assessed whether any of the double-peptide insertions resulted in higher binding affinity for the $\alpha_v\beta_3$ receptor using the solid-phase binding assay. The results revealed that the combination of insertions at positions 265–266 and 78–79 gave rise to the highest level overall of binding (Table 4). However, despite any improved affinity for the $\alpha_v\beta_3$ integrin, we were still unable to demonstrate transduction of M21 cells for any of the double-insertion chimeras (data not shown).

Taken together, these results indicate that peptide insertions are well tolerated at combinations of sites 6–7, 78–79, and 265–266, which can be used to generate multivalent chimeric Env proteins that retain full function on cells expressing the ecotropic receptor. Furthermore, the combination of the 78–79 insertion site with 265–266 enhanced the overall affinity of the chimeric Env protein for the $\alpha_v\beta_3$ integrin target receptor.

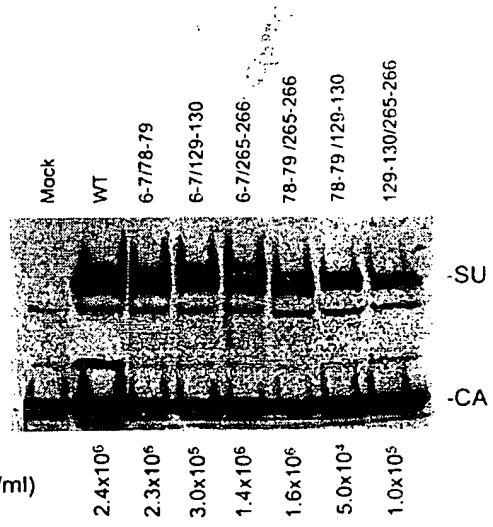

FIG. 5. Chimeric Env proteins with double-peptide insertions in the SU protein. Retroviral vectors expressing chimeric Env proteins were produced at 37°C and analyzed by Western blotting, as described in the legend to Fig. 2. The retroviral vectors were titered on NIH 3T3 cells, as shown at the bottom of the figure.

TABLE 4

Binding of Chimeric Env Proteins with Double-Peptide Insertions to Integrin $\alpha_v\beta_3$

Env protein	Binding to $\alpha_v\beta_3$[a]	
	Experiment I	Experiment II
wt	0.101	ND
6–7	0.30	0.348
78–79	0.336	0.508
91–92	0.337	0.260
129–130	0.24	0.174
265–266	0.924	0.902
6–7/78–79	0.391	0.426
6–7/129–130	0.294	0.252
6–7/265–266	0.890	1.161
78–79/129–130	0.225	0.488
78–79/265–266	1.314	1.059
129–130/265–266	0.260	0.596

[a] Binding of Env proteins to integrin $\alpha_v\beta_3$ was estimated by a solid-phase binding assay. The numbers represent the optical density at 490 nm. The results from two independent experiments are shown.

DISCUSSION

We have undertaken a comprehensive screen to identify sites in the RBD of the MoMuLV Env protein that can accommodate the insertion of a targeting peptide. The choice of sites was based on consideration of the three-dimensional structure of the highly homologous RBD of FrMuLV (Fass *et al.*, 1997), together with data from previous attempts to engineer MoMuLV Env (Russell *et al.*, 1993; Wu *et al.*, 1998). A 15-amino-acid RGD-containing peptide was inserted into various locations in the SU protein predicted to be surface exposed in the RBD monomer and therefore considered more likely to tolerate such insertions. The majority of sites chosen resided in the three variable regions of VRA, VRB, and VRC, which naturally vary in sequence between the different MuLV subtypes and are the major determinants of receptor choice (Battini *et al.*, 1992). Furthermore, we concentrated in particular on regions surrounding the predicted receptor-binding site in the VRA of the ecotropic Env (MacKrell *et al.*, 1996; Fass *et al.*, 1997). Despite these considerations, the majority of the peptide insertions we made resulted in chimeric Env proteins that were not properly processed or able to be incorporated into viral particles. These results emphasize the difficulties inherent in engineering a complex oligomeric protein, even when structural information is available.

Eight sites were identified that did tolerate the insertion of the RGD peptide. At the N-terminus of the protein, an insertion between amino acids 6 and 7 resulted in an Env protein that retained wild-type function when incorporated into retroviral vectors. The N-terminus of the SU protein, and in particular residue H8, is important for postbinding functions (Bae *et al.*, 1997; Burke *et al.*, in preparation; Zavorotinskaya and Albritton, 1999) and although single-chain antibodies can be inserted at this site (Russell *et al.*, 1993; Benedict *et al.*, 1999; Zhao *et al.*, 1999), the resulting chimeras frequently have impaired function. The retention of wild-type function by the 6–7 insertion indicates the advantage of using small peptide insertions over larger targeting ligands.

We made several insertions in the VRA region as we reasoned that the use of the presumed authentic receptor-binding site would be more likely to trigger the subsequent downstream events necessary to catalyze virus–cell fusion. VRA contains an extended region that projects away from the body of the protein and consists of two loops (MoMuLV residues 73–81 and 89–96), separated by a small helix (residues 83–87) (Fass *et al.*, 1997) that contains the key receptor-binding residue, D84 (MacKrell *et al.*, 1996). Although no individual residues in the extended VRA region (apart from the structurally important cysteines) have been identified that interfere with Env processing (MacKrell *et al.*, 1996; Davey *et al.*, 1998), the replacement of residues 74–80 or 74–84 by the peptide was not tolerated. In contrast, we observed that the peptide could successfully be inserted between residues 78 and 79, which is at the apex of this region and extends about 9 Å from the body of the protein. At other sites in VRA, we found that an insertion between residues 83 and 84, which would place the peptide directly at the predicted receptor-binding face, was not tolerated. In contrast, a site in the second loop region of VRA between amino acids 91 and 92 that tolerated the insertion of the peptide was identified, although the effects of this insertion on both titer and binding to NIH 3T3 cells suggest that the inserted peptide could be blocking the receptor-binding site. Indeed, residue L92 is part of the hydrophobic pocket predicted to be part of the receptor-binding surface in the ecotropic Env (Fass *et al.*, 1997).

We also identified a cluster of sites in VRC (residues 125–130) that tolerated the insertion of the peptide, although the resulting Env proteins were defective in binding to the ecotropic receptor. These observations were somewhat unexpected given the distance of this region from the presumed receptor-binding site and the lack of any previous data from mutagenesis studies implicating this region (MacKrell *et al.*, 1996; Burke *et al.*, in preparation). However, it has been suggested that VRC might participate in a receptor-binding site formed by oligomers of the Env protein and a speculative structure of a possible RBD trimer places VRC from one monomer close to VRB in an adjacent monomer (Fass *et al.*, 1997). Furthermore, the location of VRC at a subunit interface may explain why these insertions adversely affected the processing of Env.

The final site that we identified that could tolerate peptide insertions was in the PRR. We have previously demonstrated that a collagen-binding peptide could be inserted here without adversely affecting Env function

(Wu *et al.*, 1998) and we now extend those findings to demonstrate that a different peptide can also be tolerated. In addition, a recent report from Kayman *et al.* (1999) has demonstrated that the PRR can also tolerate the insertion of an scFv, indicating the general utility of this site for peptide insertions. Peptides inserted at different sites in Env bound to $\alpha_v\beta_3$ with different affinities, consistent with their different abilities to be recognized by an anti-peptide antibody and presumably corresponding to the extent of their surface exposure. The PRR insertion (265–266) displayed the highest level of binding and had the greatest recognition by the anti-peptide antibody. The formation of a relatively extended structure in the PRR has been suggested (Fontenot *et al.*, 1994), which could account for these properties.

Overall, our data suggest that the sites in the SU protein most able to tolerate a peptide insertion are located in regions of the protein that do not interact extensively with the rest of the SU protein. Residues 78 and 79 and 125 to 130 are located in regions that protrude from the RBD core by about 9 to 10 Å, and while the exact positioning of the 6–7 and 265–266 insertion sites is unknown, it is likely that they are also in more flexible regions, distant from the core of the envelope protein. Taken together, this indicates that exposure on the protein surface may be insufficient to create an optimal peptide insertion site, but that a lack of interaction with the body of the protein may also be required.

Some of the chimeric envelope proteins, especially those with insertions in the VRC region, showed very limited levels of binding to NIH 3T3 cells but retained a high level of infectivity when titered on NIH 3T3 cells. It is likely that this apparent discrepancy merely reflects the lower sensitivity of the binding assay than the titer assay. Alternatively, it is also possible that peptide insertions in these regions affected the ability of the anti-Env antibody to recognize the chimeric envelope proteins without inhibiting envelope protein function. In addition, some of the fusion proteins, such as 78–79, 91–92, and 125–126, were poorly processed in cells, yet were efficiently incorporated into virions. It is likely that the high level of envelope proteins resulting from our transfection procedure allows sufficient processed envelope proteins to reach the cell surface and to be incorporated into viral particles, despite these defects.

We attempted to transduce the M21 human melanoma cell line, which expresses high levels of $\alpha_v\beta_3$, with the chimeric Env proteins. Although vectors expressing the amphotropic MuLV Env protein transduced these cells reasonably efficiently, none of the chimeric Env proteins were able to transduce the M21 cells. There are two possibilities to account for this lack of success. First, the ability of the peptides to bind to $\alpha_v\beta_3$ may not be sufficient to allow efficient transduction. The peptide ligand alone has only a modest affinity for $\alpha_v\beta_3$, with an estimated IC_{50} of around 400 nm (Healy *et al.*, 1995), and additional constraints in the context of the ecotropic Env backbone might be expected to further interfere with its binding ability. Indeed we were unable to demonstrate binding to M21 cells for any of the chimeras except for the 265–266 insertion. Alternate approaches that may produce better results include the use of higher affinity ligands or by flanking the ligands with cysteine residues to facilitate a more exposed and constrained structure through disulfide-bond formation. However, even if high-affinity binding could be obtained, it remains a distinct possibility that this will not lead to the fusion of viral and cellular membranes. Indeed, we have previously demonstrated that the block to transduction for chimeric Env proteins containing insertions of scFvs is due to the inability of the scFv–receptor interaction to trigger the Env protein to a fusogenic state (Benedict *et al.*, 1999; Zhao *et al.*, 1999).

Nevertheless, the sites in the SU protein that we have identified that tolerate the insertion of a targeting peptide may have utility for targeting retroviral vectors through alternate approaches, such as "tethering" strategies. Here, the role of the targeting peptide is to concentrate the vectors at a specific target tissue, with entry subsequently proceeding through the natural Env–receptor interaction. We have previously demonstrated the utility of this approach in rodent cells using collagen-targeted ecotropic Env proteins (Hall *et al.*, 1997).

MATERIALS AND METHODS

Cell lines. 293T cells were obtained from the American Type Culture Collection (ATCC) (CRL 11268). NIH 3T3 and 293T cells were maintained in Dulbecco's modified Eagle's medium, supplemented with 10% fetal calf serum (FCS) (HyClone, Logan, UT) and 2 mM glutamine (Gibco BRL, Grand Island, NY). The M21 cell line (Cheresh and Spiro, 1987) is a human melanoma cell line kindly provided by Dr. Peter Brook (University of Southern California). The cells were propagated in RPMI 1640 medium (Gibco BRL), supplemented with 10% FCS and 2 mM glutamine. MG-63 cells (ATCC, CRL-1427) are a human osteosarcoma cell line (Stuvier *et al.*, 1996) and were maintained in minimal essential medium supplemented with nonessential amino acids (Gibco BRL) and 10% FCS.

Envelope protein expression plasmids. Plasmid pCEE+ is an expression plasmid for the MoMuLV ecotropic envelope protein (MacKrell *et al.*, 1996); plasmid E/A-PRR is an expression plasmid for MoMuLV Env but with the PRR sequence replaced by the amphotropic 4070A MuLV sequence (Wu *et al.*, 1998). The 15-amino-acid peptide sequence GERGDGSFFAFRSPF (Healy *et al.*, 1995) was inserted into various positions in the envelope protein by recombinant PCR splice overlap extension (Ho *et al.*, 1989). All chimeric Env proteins were confirmed by DNA sequencing.

Retroviral vector production. Retroviral vectors were produced by transient transfection of 293T cells with the MoMuLV Gag-Pol expression plasmid pHIT60 (Soneoka *et al.*, 1995), the retroviral vector pCnBg (Han *et al.*, 1997), which expresses *LacZ* and *neo*^{r}, and an Env expression plasmid, essentially as described (Soneoka *et al.*, 1995). Transfection was carried out by the calcium phosphate precipitation method, using a calcium phosphate mammalian cell transfection kit (5Prime → 3Prime, Inc., Boulder, CO). Approximately 16 h posttransfection, the precipitate was removed, and medium containing 10 mM sodium butyrate (Sigma, St. Louis, MO) was added to the cells for 10 h. The cells were then incubated for a further 12 h in fresh medium at 37°C, or for 48 h at 32°C, before the culture supernatant was harvested. Retroviral vectors were also produced in serum-free medium at 32°C by incubation in IS 293 (Irvine Scientific) after the sodium butyrate treatment. Vector supernatants were filtered through 0.45-μm filters and either used immediately or stored at −70°C.

Titer determination. Vector titer on NIH 3T3 cells was determined by scoring the number of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal)-positive foci following transduction of the cells. NIH 3T3 cells (3×10^4) were seeded in 3 ml of medium per 30-mm well in six-well plates and 18 h later, the medium was replaced with 1 ml of appropriately diluted viral supernatant containing polybrene (8 μg/ml) (Sigma) and incubated for 2 h at 37°C. An additional 2 ml of medium was then added, and the cultures were incubated for another 48 h, after which the cells were stained by X-gal to detect β-galactosidase expression as previously described (MacKrell *et al.*, 1996) and the number of blue colonies was determined. Titer was expressed as colony-forming units per milliliter.

Vector titer on human cells was determined by scoring the number of neomycin-resistant colonies. High-level expression of the $\alpha_v\beta_3$ integrin on the cell surface of the M21 and MG-63 cell lines was confirmed by fluorescence-activated cell sorting (FACS) analysis using monoclonal antibody LM609 (Chemicon, Temecula, CA). The cells were seeded at 3×10^4 cells per 30-mm well in six-well plates and 18 h later the medium was removed and the cells were incubated in IS 293 medium at 37°C for 20 min. The cells were then incubated with viral supernatants harvested at 32°C in IS 293 medium in the presence of 4 μg/ml protamine sulfate (Sigma) for 3 h before the addition of 2 ml RPMI 1640 medium. Twenty-four hours later, the medium was replaced with fresh medium containing 0.5 mg/ml of G418 (Gibco BRL) in order to select for transductants. Viral titer was scored by the number of neomycin-resistant colonies established after 10 days of selection.

Western blot analysis of Env proteins. Retroviral particles generated by transient transfection of 293T cells were pelleted through 20% sucrose at 16,000 *g*, 4°C for 30 min. Viral pellets were resuspended in 30 μl of 2× SDS gel loading buffer (100 mM Tris–HCl, pH 6.8, 4% SDS, 20% glycerol, 0.01% bromphenol blue, 1.4 M β-mercaptoethanol) and boiled for 5 min. Viral protein samples were resolved on a precast 8–16% polyacrylamide gel (Novex, San Diego, CA) and transferred onto an Immobilon membrane (Millipore, Bedford, MA). The blot was blocked overnight at 4°C in 5% powdered milk in TBS buffer (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 0.25% Tween 20) and incubated with primary antibodies at room temperature for 2 h. After being washed in TBS buffer for 30 min, blots were incubated with secondary antibodies for 1 h at room temperature followed by a further 30-min washing in TBS. Specific proteins were visualized using the ECL detection system (Amersham International plc., Arlington Heights, IL). The primary antibodies used were goat anti-Rauscher MuLV gp69/71, 1:3000 dilution (Quality Biotech, Camden, NJ; Lot 79S656); goat anti-Raucher MuLV p30, 1:10,000 dilution (Quality Biotech, Lot 78S221), and rat anti-AKR MuLV p15E, 1:2000 dilution (BABCO, Berkeley, CA, Lot 42/114) (Pinter and Fleissner, 1977). The secondary antibodies were horseradish peroxidase (HRP)-conjugated rabbit anti-goat immunoglobulin G (IgG) (1:10,000) and HRP-conjugated goat anti-rabbit IgG (1:10,000) (Pierce, Rockford, IL).

To analyze the form of the envelope proteins present in cell lysates, 293T cells were transfected with envelope protein expression plasmids alone (30 μg per 10-cm plate) and lysates were prepared by incubating the cells in 500 μl lysis buffer (20 mM Tris–HCl, pH 7.5, 1% Triton X-100, 0.05% SDS, 5 mg/ml sodium deoxycholate, 150 mM NaCl, and 1 mM phenylethanolamine fluoride) for 10 min at 4°C, followed by centrifugation at 10,000 *g* for 10 min to pellet nuclei. Envelope proteins were denatured by treating the lysates with 0.5% SDS and 1% β-mercaptoethanol at 100°C for 5 min and then deglycosylated with 50 mM sodium phosphate (pH 7.5), 1% NP-40, and 500 units *N*-glycosidase F (New England Biolabs, Beverly, MA) at 37°C for 1 h. Envelope proteins were detected by Western blotting as described above.

Flow cytometry analysis of Env proteins on cell surface. Env protein on the cell surface was measured by FACS of 293T cells that transiently expressed the wild-type or chimeric Env proteins. 293T cells in a 10-cm plate were transfected with 30 μg of envelope protein expression plasmid and 48 h later the cells were harvested with trypsin-free cell dissociation buffer (Gibco BRL). The cells were washed with 10% goat serum in phosphate-buffered saline (PBS), followed by incubation for 1 h at 4°C with 250 μl of undiluted hybridoma supernatant containing monoclonal antibody 83A25 (Evans *et al.*, 1990). After incubation, the cells were washed again and resuspended in 100 μl of 1:100-diluted fluorescein isothiocyanate (FITC)-labeled goat anti-rat IgG (Kirkegaard

& Perry Laboratories, Inc., Gaithersburg, MD) for continued incubation for 1 h at 4°C. Cells were then washed and fixed in 4% paraformaldehyde in PBS. The level of envelope protein expression on the cell surface was estimated by measuring the fluorescence intensity of the samples with a FACStar Plus flow cytometer (Becton Dickinson, San Jose, CA).

Flow cytometry analysis was also used to examine the accessibility of the inserted RGD peptide to antibody. The chimeric Env proteins were expressed on the surface of 293T cells as described above and the cells were incubated with a rabbit antiserum raised against the RGD peptide (derived by Zymed, San Francisco, CA). The secondary antibody used was FITC-labeled goat anti-rabbit IgG (Kirkegaard & Perry Laboratories, Inc.).

Binding of viral particles to NIH 3T3 cells. To measure the binding of viral particles to NIH 3T3 cells, the cells were dissociated by trypsin treatment and washed in 10% goat serum in PBS, and 5×10^5 cells were mixed with 1 ml of retroviral vector supernatant (harvested at 32°C) and incubated at 4°C for 2 h with gentle rotation. The cells were then washed in 10% goat serum in PBS twice and the bound viral particles were detected by flow cytometry analysis, as described above for the detection of cell surface Env.

Solid-phase binding assay. Plastic 96-well plates were coated with $\alpha_v\beta_3$ integrin (1 μg/ml) (Chemicon, Temecula, CA) by being incubated in coating buffer (20 mM Tris–HCl, 150 mM NaCl, 0.5 mM $CaCl_2$, 1.0 mM $MgCl_2$, 1.0 mM $MnCl_2$, pH 7.5) overnight at 4°C. The wells were washed twice with PBS and blocked with 1% casein (Sigma) in PBS at 37°C for 1 h and then washed twice with binding buffer (50 mM Tris–HCl, 150 mM NaCl, 0.05 mM $CaCl_2$, 1.0 mM $MgCl_2$, 1.0 mM $MnCl_2$, pH 7.5). Viral particles were collected in serum-free medium at 32°C, purified by pelleting through 20% sucrose, and resuspended in 50 μl binding buffer. This was added to the wells and incubated for 1 h at room temperature. After two washes with binding buffer, the bound viral particles were detected by an ELISA. In brief, the wells were incubated with a 1:5 dilution of rat monoclonal antibody 83A25 in 0.5% casein (w/v) at 37°C for 1 h. After being washed twice in washing buffer (10 mM Tris–HCl, pH 8.0, 138 mM NaCl), the wells were incubated with HRP-conjugated goat anti-rat IgG diluted 1:2000 in 0.5% casein at 37°C for 1 h and then washed twice with washing buffer. Finally, 50 μl of *o*-phenylenediamine dihydrochloride in 24 mM citric acid and 50 mM dibasic sodium phosphate was added to the wells, and the color was allowed to develop for 5 min at room temperature and stopped by the addition of 25 μl of 4 M hydroxy sulfate. The color was monitored at 490 nm with an Emax precision microplate reader (Molecular Devices, Sunnyvale, CA).

ACKNOWLEDGMENTS

We thank Dr. Peter Brook for helpful discussion. This work was supported by Genetic Therapy, Inc./Novartis and Public Health Service Grant CA-59318 from the National Cancer Institute.

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RESEARCH NEWS

Short-order Sindbis vector targeting

Tom Wickham

Let's say you were interested in targeting a therapeutic gene to a specific cell type. Wouldn't it be nice if you were able to simply order an antibody from your favorite catalog, mix the antibody with your gene delivery vector, and—voila! Target your vector to the correct cell. Until recently, that was only a dream to those interested in receptor-targeted gene delivery. However, that is just the system reported in this issue by Meruelo and colleagues[1], using a Sindbis viral vector.

They describe the design and production of a Sindbis virus vector comprising a chimeric E2 viral envelope and an optimized IgG binding domain of *Staphylococcus aureus* protein A. The insertion of an optimized protein A sequence into the E2 envelope protein gene blocked binding of the virus to its native high-affinity laminin cellular receptor. The virus was then specifically targeted to different cellular receptors by mixing different receptor-specific IgG monoclonal antibodies with the chimeric Sindbis virus (see Fig. 1). The authors demonstrated the versatility of the system by targeting the virus to transduce cells expressing the cellular receptors CD4, CD33, or human leukocyte antigen (HLA) via antibodies directed toward these molecules.

Targeted gene therapy has been pursued by a number of academic and industrial research groups. An entire Cold Spring Harbor conference[2] was recently devoted to vector targeting strategies for therapeutic gene delivery. In general, groups have attempted to target viral vectors through the genetic incorporation of the targeting moiety into the virus coat[3,4] or by covalently/noncovalently linking the targeting moiety to the viral vector[5,6].

The principal problems encountered in targeting gene delivery have been loss of the viral vectors' original transduction efficiency, ablation of virus binding to its native receptor, and difficulties encountered in incorporating targeting moieties into the virus coat and/or in synthesizing and characterizing exogenously added targeting moieties.

For example, groups trying to target retrovirus have been hampered by the often dramatic reductions in transduction efficiency when new ligands are added to the retrovirus coat proteins[7]. These reductions in efficiency may result from the interference of the targeting ligand with the normal entry mechanism of retrovirus. In targeting adenovirus, transduction efficiency does not appear to be severely affected by the targeting moiety. However, ablation of native adenovirus receptor binding, as well as the ease of synthesizing exogenous targeting moieties, have been somewhat problematic.

The use of bispecific molecules that both neutralize native receptor binding and redirect virus binding to a targeted receptor has been successful[5]. However, production, purification, and characterization of the bispecific molecule, as well as the quantities of ligands that are often required for chemical crosslinking, are cumbersome obstacles to rapidly assessing targeting strategies.

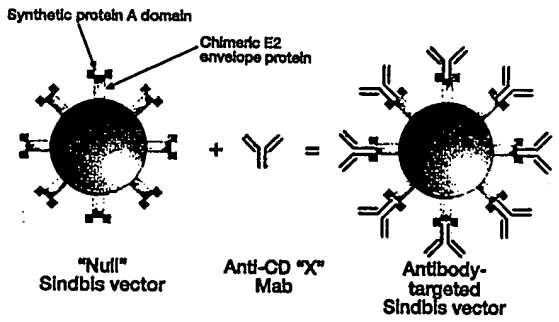

Figure 1. Chimeric Sindbis vectors can be targeted to cells "to order" using monoclonal antibodies (Mabs) against receptors of interest (CD "X").

The approach successfully used for Sindbis virus largely avoids the difficulties above. It is possible to target the vector using small amounts of unmodified, commercially available antibodies by creating a single, "null" vector that lacks native receptor binding and that is capable of binding IgG antibodies. Using this vector, it now should be possible to screen many receptors for their usefulness in targeted gene delivery without significant decreases in transduction efficiency, the presence of native receptor binding, and the need for bispecific targeting moieties.

Despite the ease and simplicity of the Sindbis system, numerous issues remain unresolved. The Sindbis system is rather "off-the-beaten-path" of vectors commonly employed in virus-mediated gene therapy, such as adenovirus, adeno-associated virus, retrovirus, and lentivirus. As such, this vector system is not without its recognized, and as-yet-unrecognized, difficulties. In its current form, the vector is known to cause apoptosis and cell death in transduced cells. However, manipulations of the genome, as well as the development of suitable packaging cell lines, could overcome this limitation.

Another current drawback of the vector is that it is an enveloped RNA virus in which production of viable vectors requires transfection of helper plasmids into packaging cells. There is also a significant, unexplained reduction in viral titers on some of the targeted cell lines. Finally, Meruelo and colleagues only report in vitro data using their system, so the effectiveness of the system in vivo is unclear. For example, it is not known whether the IgG–protein A affinity is high enough to keep the antibodies associated with the virus in vivo. Also, the presence of the Fc regions on the IgG may complicate targeting and activate immune clearance mechanisms through binding to Fc receptors.

Regardless of these hurdles, the greatest advantage of this vector targeting system is its ease and simplicity. As a tool, the system will be quite useful in rapidly assessing different tissue-specific targeting strategies to be used in gene delivery. With further modifications to the system, it should also be possible to genetically incorporate specific targeting domains into the virus. Such targeting domains could include portions of receptor ligands, high affinity peptide-binding motifs, or single chain antibodies. The results of such studies will not only be useful for those working with individual vector targeting systems, but especially for those interested in applying gene targeting technology to the treatment of human disease.

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Cell-specific targeting of Sindbis virus vectors displaying IgG-binding domains of protein A

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Received 18 March 1997; accepted 5 June 1997

Sindbis virus can infect a broad range of insect and vertebrate cell types due to the widespread distribution of the cellular receptor for the virus. The development of Sindbis virus vectors that target specific cell types could have important implications for the design of gene therapy strategies. To achieve this goal we have designed and constructed Sindbis virus particles displaying the IgG-binding domain of protein A. The protein A-envelope chimeric Sindbis virus vector has minimal infectivities against baby hamster kidney and human cell lines. When used in conjunction with monoclonal antibodies that react with cell-surface antigens, however, the protein A-envelope chimeric virus was able to infect human cell lines with high efficiency. Infection rates were 90% or higher for human lymphoblastoid cells. A variety of cells could be targeted by changing the monoclonal antibody without generating a new recombinant virus.

Keywords: gene therapy vector, alphavirus, targeting, protein A

Sindbis virus, a member of the *Alphavirus* genus, has received considerable attention for use as virus-based expression vectors. Many properties of alphavirus vectors make them a desirable alternative to other virus-derived vector systems being developed, including rapid engineering of expression constructs, production of high-titered stocks of infectious particles, infection of nondividing cells, and high levels of expression[1–4]. However, a major drawback to the use of Sindbis virus vectors is that these vectors lack target-cell specificity. For mammalian cells, at least one Sindbis virus receptor is the high-affinity laminin receptor, whose wide distribution and highly conserved nature may be in part responsible for the broad host range of the virus[1,5]. It is desirable to alter the tropism of the Sindbis virus vectors to permit gene delivery specifically to certain target cell types. This will require both the ablation of endogenous viral tropism and the introduction of novel tropism. In the mature Sindbis virion, a plus-stranded viral genome RNA is complexed with capsid protein C to form icosahedral nucleocapsid that is surrounded by lipid bilayer in which two integral membrane glycoproteins, E1 and E2, are embedded[1]. Although E1 and E2 form a heterodimer that functions as a unit, the E2 domain appears to be particularly important for binding to cells. Monoclonal antibodies (Mabs) capable of neutralizing virus infectivity are usually E2-specific[6,7], and mutations in E2, rather than E1, are more often associated with altered host range and virulence[8,9]. A Sindbis virus mutant was identified with an insertion in E2 resulting in defective binding to mammalian cells[10].

Several attempts to alter the host range of viruses have been reported to date. For retrovirus-based vectors, direct modifications of the envelope protein of murine leukemia virus (MLV) redirects viral tropism. A recombinant virus containing a fragment encoding a single Fv antibody chain at the N-terminal region of the MLV *env* gene has been shown to recognize the corresponding epitopes[11] and infect human cells[12,13]. Kasahara et al.[14] have made a chimeric ecotropic virus containing an erythropoietin-envelope fusion protein. This chimeric virus has been shown to infect human cells bearing the erythropoietin receptor. In these systems, however, each targetable vector much be constructed de novo, and only very low levels of infectivity were observed[13,14]. Furthermore, virions constructed to directly bind to specific targets in human cells are intrinsically unsafe, as wild-type recombinants could possibly go on to cause cancer in patients treated with such vectors.

We describe the construction of a recombinant Sindbis virus vector displaying protein A (PA)-envelope chimeric proteins to redirect the viral tropism. PA, derived from *Staphylococcus aureus*, has a strong affinity for the Fc region of various mammalian IgGs[15]. In contrast to the targeted retroviral vectors described above, the PA-envelope chimeric virus vector, once successfully generated, needs no further modification to target distinct cells. The targeting is achieved simply by changing the complementary Mab (Fig. 1A). More importantly, we demonstrate that this chimeric virus used in conjunction with Mabs can infect human cells and transfer a marker gene, bacterial β-galactosidase, with high efficiency.

Results

Construction of PA-envelope Sindbis virus helper plasmid. To modify the Sindbis virus envelope protein, the DH-BB helper plasmid (Fig. 1B), constructed by deletion of the region between BspMII and BamHI sites of the full-length Sindbis virus cDNA clone[16], was utilized. When RNA from DH-BB is cotransfected with recombinant RNA from the Sindbis virus expression vector, the structural proteins expressed in trans, from the DH-BB RNA transcript allows packaging of the recombinant RNA into virions. As DH-BB does not contain a packaging signal, it will not form a defective interfering particle or be packaged with recombinant RNA. Two modified Sindbis virus helper plasmids were constructed: DH-BB-Bst into which a BstEII cloning site was inserted, and DH-BB-ZZ into which two IgG-binding domains of PA were inserted in the E2 region (Fig. 1B). Native PA has five homologous IgG-binding domains (E, D, A, B, and C). We used the synthetic Z domain, which is based on the B domain of protein PA (ref. 17). The insertion position, between codons 71 and 74 amino acids in E2, was

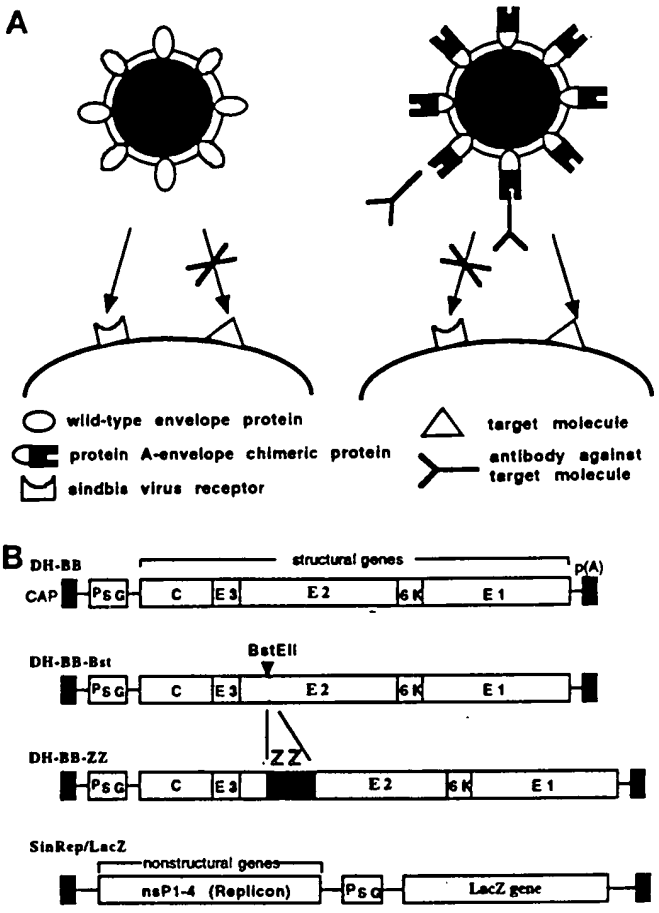

Figure 1. (A) Strategy for retargeting a Sindbis virus vector. A wild-type Sindbis virus (left) binds to mammalian cells via its conserved surface receptor. A recombinant Sindbis virus displaying the IgG-binding domain of protein A (right) permits binding to a novel target molecule on the cell surface when used with a corresponding monoclonal antibody. (B) Schematic representation of recombinant helper constructs and a SinRep/LacZ expression vector. DH-BB is a parental helper plasmid that contains the genes for the structural proteins (capsid, E3, E2, 6K, and E1) required for packaging of the Sindbis viral genome. DH-BB-Bst contains a BstEII cloning site between amino acids 71 and 74 of the E2 glycoprotein. DHBB-ZZ contains the synthetic IgG-binding domain (ZZ) of PA at the BstEII site in DH-BB-Bst. SinRep/LacZ contains the packaging signal, nonstructural protein genes for replicating the RNA transcript and lacZ gene. P_{SG}: Sindbis viral subgenomic promoter; C: capsid; nsP1-4: nonstructural protein genes 1–4; ZZ: synthetic IgG-binding domain of protein A; p(A): polyadenylation signal.

chosen because mutations in this region allow normal particle assembly and release and block virus entry at the level of attachment[16].

Expression and incorporation of chimeric envelopes into virions. After linearization of helper and SinRep/LacZ plasmids, in vitro transcription was performed and the quality of RNA was checked on agarose gels (data not shown). To examine the expression of the recombinant envelope, recombinant helper RNA was cotransfected with RNA from SinRep/LacZ plasmid into baby hamster kidney (BHK) cells by electroporation. The transfection efficiency was usually nearly 100% (data not shown). Lysates from transfected cells were first analyzed for expression of structural protein by using anti-Sindbis virus immune ascitic fluid. DH-BB-Bst helper RNA expressed a 50 to 55 kDa envelope (E1 and E2) and a 33 kDa capsid protein, which is the same protein profile as the parental virus produced by DH-BB (Fig. 2A). A band of 60 kDa corresponding to the E2 precursor PE2 was also detected. In the protein profile expressed by DH-BB-ZZ RNA, a major band between 65 to 70 kDa, which is the estimated molecular weight of PA-E2 and PA-PE2 chimeric protein, was observed as well as the 33 kDa capsid protein. These results suggest that the mutants were correctly expressed and processed.

Virions produced by DH-BB and DH-BB-Bst RNA contain capsid and envelope (E1 and E2) proteins indicating that the mutation in DH-BB-Bst does not affect virus assembly (Fig. 2B). The PA-E2 chimeric protein was also incorporated into virions and exhibited IgG-binding activity, which is not detected in that of DH-BB and DH-BB-Bst (Fig. 2B and C). These results demonstrate that DH-BB-ZZ produces recombinant Sindbis pseudovirions displaying the IgG-binding domain in its envelope. The E1 protein, which was expressed in transfected cells (Fig. 2A), could not be detected in the virions produced by DH-BB-ZZ RNA. This may be because the antisera being used using reacts poorly—or not at all—with the E1 protein. The gels used may not adequately separate E1 and E2. While we have assumed that the band running around 50k PA is E1, this band may represent a small amount of E2 in which the PA insert has been deleted. E1 migrates slower than E2 and the presumptive E1 observed is migrating faster than expected. It is also possible that the antisera used does recognize E1. In this case, the effect could be due to loss of a sugar group in E1 through lack of chaperoning by E2, which could be one reason for misfolding of E1 and reduced incorporation into virions.

Infection with viruses carrying mutant envelopes. Recombinant virus infectivity of hamster and human cells was determined by transfer of the Sindbis virus vector (SinRep/LacZ) that can transduce bacterial β-galactosidase gene. Viruses derived from DH-BB and DH-BB-Bst helper showed very high infectious titer (10^6 LacZ CFU/ml) against BHK cells whereas viruses produced by DH-BB-ZZ showed very low infectivity (10^3 LacZ cfu/ml) suggesting that the protein A insertion into E2 blocked virus binding to host cells sup-

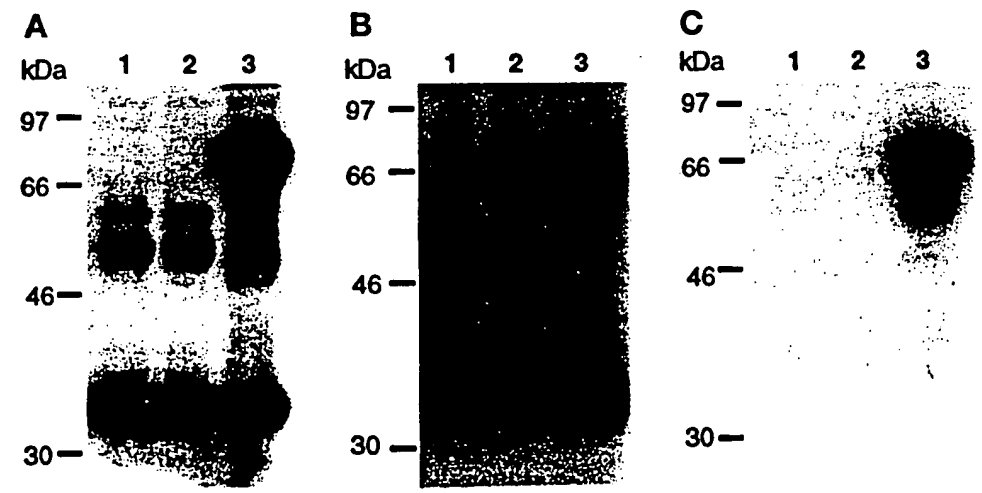

Figure 2. Western blot analysis of Sindbis viral structural protein components (A). Cell lysates from BHK cells transfected with helper RNA and (B and C) pellets of viral particles produced from these cells. Viral proteins were stained with diluted anti-Sindbis virus mouse immune ascitic fluid to detect all structural components (A and B) or with HRP-conjugated goat anti-mouse IgG to detect protein A-envelope chimeric protein (C). Lane 1: DH-BB; lane 2: DH-BB-Bst; lane 3: DH-BB-ZZ.

porting previous observations[10] (Table 1). The PA-envelope virus also showed minimal titer against human HeLa-CD4⁺ cells (10^2 LacZ cfu/ml). When virions were preincubated with anti-CD4 Mab, however, the PA-envelope chimeric virus could infect HeLa-CD4⁺ cells in an antibody dose-dependent manner (Table 1). When the viral supernatant was preincubated with 0.5 μg/ml Mab, the infectious titer was approximately 2.3×10^5 LacZ cfu/ml. The enhancement of infectivities by Mab was not observed with DH-BB- and DH-BB-Bst derived viruses. As determined by enzyme-linked immunosorbant assay (ELISA) using anti-Sindbis antibodies, the ratio of DH-BB to DH-BB-ZZ was slightly greater than to 1:1. When the ratio of virus particles, as determined ELISA assays using anti-Sindbis antibodies, is 1:1, the efficacies of infection of DH-BB and DH-BB-ZZ are comparable on HeLa-CD4⁺ cells (DH-BB is actually slightly more infectious), DH-BB-ZZ is more than 50% as infectious as DH-BB.

The PA-envelope chimeric virus with anti-CD4 Mab could not infect HeLa cells, which do not express CD4, indicating that the infection is dependent on both an antibody and a corresponding antigen (Fig. 3). These data demonstrate that the PA-E2 chimeric envelope derived from DH-BB-ZZ helper RNA can redirect Sindbis virus infection via a new receptor/antigen in the presence of recognizing antibody.

Viruses with PA-envelope could infect the adherent epidermoid carcinoma cell line A431 and glioblastoma cell line U87MG, which overexpress epidermal growth factor receptors (EGFR), only when virions were preincubated with anti-EGFR Mab (Fig. 4). Infectious titers of the recombinant virus with Mab (0.5 μg/ml) against A431 and U87MG cells were approximately 10^4 LacZ cfu/ml. Minimal infectivities (10^2 LacZ cfu/ml) were seen on these cells when infected without Mab. In contrast to adherent cells, the wild-type virus particles derived from DH-BB helper RNA have very low infectivities against the suspension cell lines and HL-60 (Fig. 5). The PA-envelope virus preincubated with corresponding Mabs (anti-HLA-DR for Daudi and anti-CD33 for HL-60), however, infects more than 90% of these cells. Infection by the PA-envelope virus of these cells was not observed in the absence of Mab.

The efficiency of infection, which varies between 20% to 90%, may depend on the receptor being targeted. Thus the efficiencies of infection for HeLa-CD4⁺, A431, and U87MG cells is somewhat lower than the efficiencies observed with Daudi or HL-60 cells.

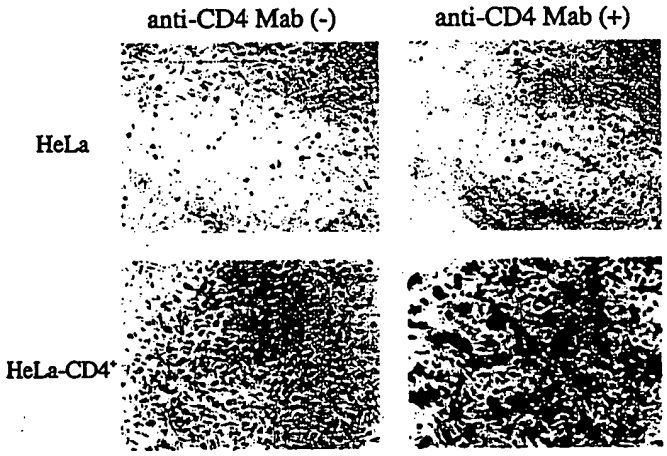

Figure 3. Infection of HeLa and HeLa-CD4⁺ cells with recombinant Sindbis virus derived from DH-BB-ZZ helper RNA. Viral supernatants (200 μl) were preincubated without or with anti-CD4 Mab (0.5 μg/ml) and added to cells (2×10^6) in 6-well plates. Viral infection was evaluated by X-Gal staining.

Discussion

In these experiments, the incorporation of E1 glycoprotein into virions could not be detected (Fig. 2C). While we believe we may be detecting E1 in transfected cells (Fig. 2A), the antisera we used may react poorly—or not at all—with the E1 protein. Further, the gels we used may not have separated E1 and E2, and the band running around 50k may represent a small amount of E2 in which the PA insert has been deleted. Further experiments will be conducted to determine whether E1 is indeed present in the virus or not, and whether it is modified in some manner (e.g., by loss of a sugar group). The interaction between E1 and E2 of alphaviruses is considered very important and E1 is deemed to play an important role in membrane fusion. A different result may provide new insights into the mechanism of Sindbis virus assembly.

The PA-envelope chimeric Sindbis virus vector showed minimal infectivities against BHK and other human cell lines. However, when used in conjunction with Mabs that react with cell surface antigens, the PA-envelope chimeric virus was able to transfer the LacZ gene into human cell lines with high efficiency. The new tropism of the recombinant virus depends on antigen-antibody interaction because the PA-envelope virus could not infect targeted cells without Mab and corresponding antigen on cell surface (Fig. 3). Taken together, the PA-E2 chimeric envelope derived from DH-BB-ZZ helper RNA can effec-

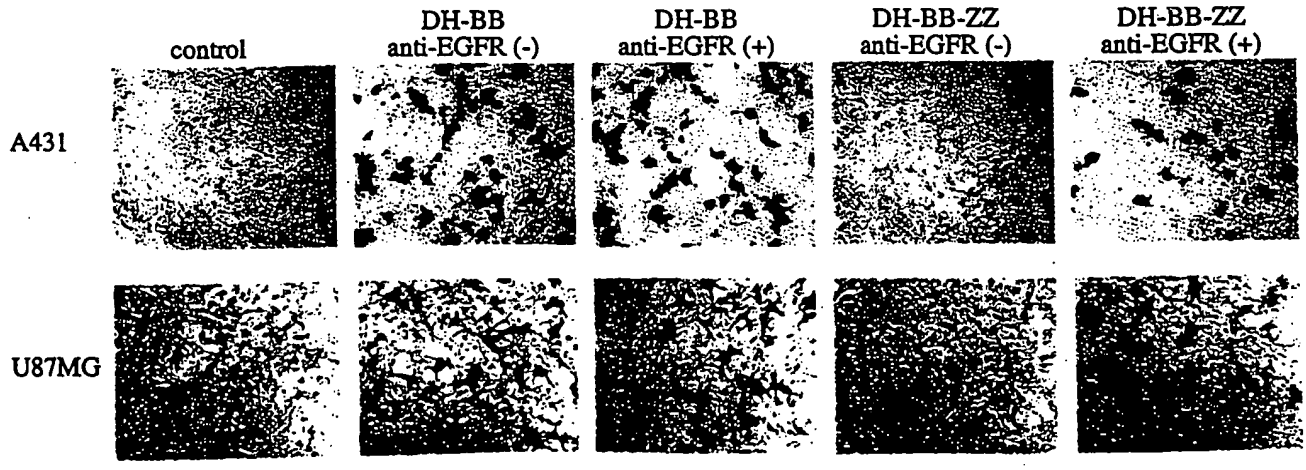

Figure 4. Antibody-dependent infectivities of recombinant Sindbis virus particles on A431 and U87MG cells. Viral supernatants (20 μl for DH-BB, 500 μl for DH-BB-ZZ) were preincubated without or with anti-EGFR Mab (0.5 μg/ml) and added to cells (2×10^6) in 6-well plates. Viral infection was evaluated by X-gal staining.

Table 1. Infection by wild-type and recombinant Sindbis virus particles*.

Cell line	Preincubation*	Titer (LacZ cfu/ml) DH-BB	DH-BB-Bst	DH-BB-ZZ
BHK	(-)	1.23×10^8	1.33×10^8	3.23×10^3
HeLa-CD4⁺	anti-CD4 Mab (ng)			
	0	1.1×106	1.0×106	<500
	20	1.1×106	N.D.	3.8×104
	100	1.2×106	N.D.	1.7×105
	500	1.0×106	1.1×106	2.3×105

*Viral supernatants derived from BHK cells transfected with DH-BB, DH-BB-Bst, and DH-BB-ZZ were preincubated without or with Mab (0 to 0.5 μg/ml) and subjected to infection of BHK and HeLa-CD4⁺ cells in serial dilutions to determine virus titer. As determined by ELISA using anti-Sindbis antibodies, the ratio of DH-BB to DH-BB-ZZ was slightly greater than to 1:1. N.D. not determined.

tively redirect Sindbis virus by antigen-antibody interaction. Using this vector we have been able to successfully infect every cell type we have tested. Generally speaking, greater than 20% of the target cell population is infected, but results of more than 90% infection are not uncommon. For some cells that are not infected by the unmodified Sindbis virus, like Daudi and HL-60, the unmodified virus infects 0% to 1.1% of the cells, whereas the PA-enveloped virus infects more than 90% of the cells. For other cell types, like HeLa-CD4⁺ cells, which can be infected by unmodified and modified Sindbis virus, the efficiency of DH-BB-ZZ is greater than 50% of DH-BB.

Several retrovirus and adenovirus-based cell-targeting vectors have been developed[11,13,14,18]. Compared with these retroviral and adenoviral retargeting vectors, in this approach it is not necessary to construct each targetable vector de novo. It is unlikely that the incorporation of different targeting elements in the envelope of the virus can always be achieved with equal success and without reducing the virus titers that could be obtained. Since the PA portion of the chimeric envelope binds to the Fc domain of the antibody[15], it allows flexibility with regards to the targeting elements, as any of a variety of Mabs can be selected. In addition, replication occurs entirely in the cytoplasm of the infected cells as an RNA molecule, without a DNA intermediate[1]. This is in contrast to retrovirus vectors, which must enter the nucleus and integrate into the host genome for initiation of vector activity. Thus, retrovirus-derived vectors have applications for long-term expression of foreign proteins, while alphavirus vectors are useful primarily for transient high-level expression. Furthermore, although adenovirus vectors can express high levels of foreign proteins, these systems are far more complex than alphaviruses and express many highly antigenic virus-specific gene products including structural proteins[19]. In contrast, current alphavirus vectors express only the four viral replicase proteins (nonstructural proteins nsP1 through nsP4) required for RNA amplification in the transduced cells.

There are several problems that we have to consider in this study. First, Sindbis virus infection of vertebrate cells usually results in cell death by apoptosis[20], with the notable exception of neuronal cells in which a persistent infection may be established[21]. Although this cytotoxicity may be suitable for gene therapy for cancer, long-term or inducible expression vectors would have broader application. Several possibilities exist for controlling this potential disadvantage, and they need to be explored. Second, the recombinant Sindbis virus vector developed in this study still has low infectivities even in the absence of antibody. There might be other sites than E2 or E1 that are involved in receptor binding[1]. Furthermore, different receptors have been identified on chicken embryo fibroblast[22] and mouse neuronal cells[23], suggesting that the virus can use more than one receptor. For safety reasons, it is necessary to develop recombinant Sindbis virus vectors that do not infect mammalian cells when not used with Mabs. Finally, the system we have described requires clean antibodies in sufficient amounts; however, we do not feel this is an obstacle to development of

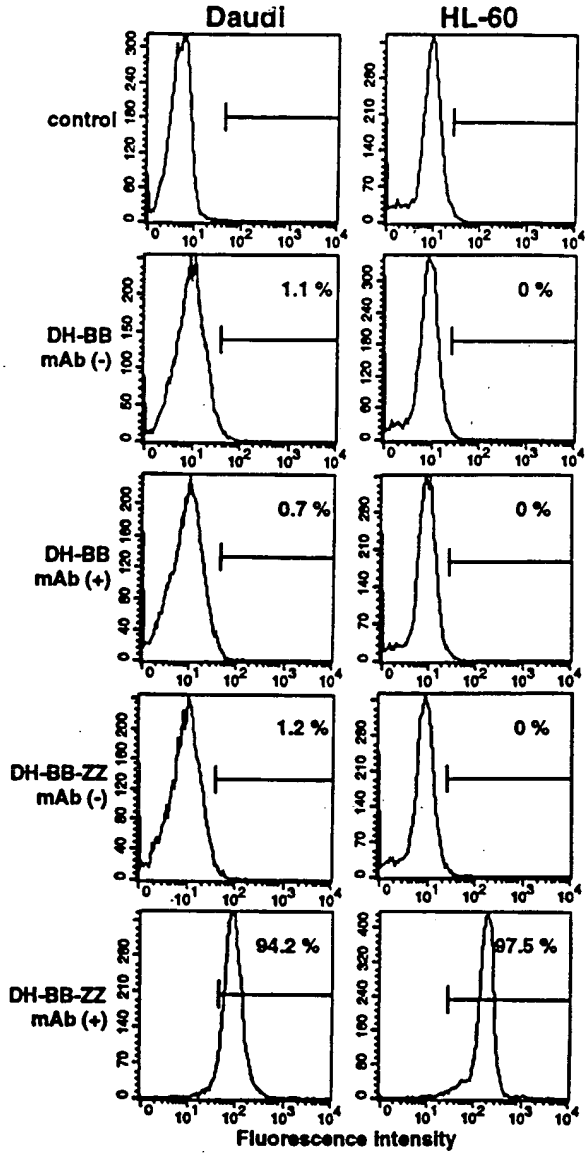

Figure 5. Antibody-dependent infectivities of recombinant Sindbis virus particles on Daudi and HL-60 suspension cell lines. Viral supernatants (500 μl) derived from DH-BB and DH-BB-ZZ transfected BHK cells were preincubated without or with 0.5 μg/ml of Mabs (anti-HLA-DR for Daudi and anti-CD33 for HL-60) and added to cells (1×10^6) in 6-well plates. Control shows uninfected cells. Viral infection was evaluated by FACS-gal analysis. Positive percent of infected cells are shown in each panel.

this system. Antibodies used for clinical imaging are produced abundantly and in very clean form, and have been shown to possess a high degree of clinical specificity. There is no reason why the system we have described cannot take advantage of the availability of these reagents.

We have retargeted a Sindbis virus vector by using the PA-antibody interaction. A similar approach may be used with other viral vectors, such as retrovirus and adenovirus vectors by inserting the synthetic IgG binding domain (ZZ) of PA. The virus-based vectors displaying PA-envelope could be very useful and have a broad applicability for gene transfer and for the gene-therapy field.

Experimental protocol

Cell lines. BHK cells were obtained from Invitrogen (San Diego, CA) and maintained in minimum essential medium alpha-modification (αMEM, JRH Biosciences, Lenexa, KS) supplemented with 5% fetal bovine serum (FBS, Gemini Bio-Products, Calabasas, CA). A human epidermoid carcinoma cell line

A431 (ATCC CRL1555), a human epitheloid carcinoma cell line HeLa (ATCC CRL2), and a human glioblastoma cell line U87MG (ATCC HTB14) were grown as monolayers in Dulbecco's modified Eagle's medium (DMEM; Gibco-BRL, Gaithersburg, MD) supplemented with 10% FBS. HeLa CD4$^-$ Clone 1022 (NIH AIDS Research and Reference Reagent Program), which expresses CD4 on its surface and a human Burkitt's lymphoma cell line Daudi (ATCC CCL213) (ATCC CRL1582) were maintained in RPMI 1640 (JRH Bioscience) supplemented with 10% FBS. HL-60, promyelocytic leukemia cell line (ATCC CCL240), was maintained in RPMI 1640 supplemented with 20% FBS.

Monoclonal antibodies (Mabs). A murine Mab of IgG2a type against the human EGFR was obtained from Upstate Biotechnology (Lake Placid, NY). Anti-HLA-DR (mouse IgG2a), anti-CD4 (mouse IgG1), and anti-CD33 (mouse IgG1) were purchased from Becton Dickinson (San Jose, CA).

Plasmids. A helper plasmid DH-BB (Invitrogen, Fig. 1B)[16] which contains the genes for the structural proteins (capsid, E3, E2, 6K, and E1) required for packaging of the Sindbis viral genome, was used for construction of the recombinant envelope gene. A Sindbis virus-based expression vector SinRep/LacZ (Invitrogen, Fig. 1B)[16] contains the packaging signal, nonstructural protein genes 1-4 (nsP1-4) for replicating the RNA transcript and the lacZ gene. Plasmid pEZZ 18, which contains two synthetic Z domains based on the B domain of PA (ref. 24), was purchased from Pharmacia Biotech (Uppsala, Sweden). The phagemid pALTER-1 vector (Promega, Madison, WI) was used to introduce the BstEII site in the E2 region of DH-BB plasmid by oligo-directed site-specific mutagenesis.

Construction of the recombinant Sindbis virus structural gene. Altered Sites in vitro Mutagenesis System (Promega) was used to introduce a specific restriction site into the E2 region of Sindbis virus structural gene. First, a BssHII site was introduced between XbaI and HindIII sites of the pALTER-1 vector by using two compatible oligonucleotides, 5'-CTAGAGCGCGCAAA-3' and 5'-AGCTTTTGCGCGCT-3'. A fragment between SacI and BssHII of the DH-BB plasmid containing the E2 region of structural gene was cloned into the pALTER-1 vector. A single-stranded template of the recombinant pALTER-1 vector was prepared by infection of helper phage M13KO7. A mutagenic oligonucleotide (5'-ATGTCGCTTAAGCAGGTAACCACCGTTAAA-GAAGGC-3'), which introduces a BstEII cloning site between codons 71 and 74 amino acids in E2 polypeptides, and an ampicillin repair oligonucleotide (5'-GTTGCCATTGCTGCAGGCATCGTGGTG-3') were annealed to the single-stranded template, followed by synthesis of the mutant strand with T4 DNA polymerase. After transformation into *Escherichia coli*, mutants were selected in the presence of ampicillin and screened by direct sequencing of the plasmid DNA. The SacI-BssHII region of original DH-BB plasmid was replaced with the mutated fragment and the DH-BB-Bst plasmid was obtained (Fig. 1B). A region of protein A (ZZ) containing two synthetic IgG-binding domains was amplified by polymerase chain reaction (PCR) using pEZZ 18 as a template. Primers used for PCR amplification are ZZ-5 (5'-CACGATGAG-GTAACCGACAACAAATTCAAC-3') and ZZ-3 (5'-GGTCGAGGTTACCG-GATCCCCGGGTACCGA-3') both encoding unique BstEII sites. The resulting PCR products were digested with BstEII and inserted into predigested DH-BB-Bst plasmid at the BstEII site. Clones containing inserts of proper size and orientation were sequenced to confirm that the correct reading frames were maintained and the DH-BB-ZZ plasmid was obtained (Fig. 1B).

In vitro transcription and transfection for recombinant virus production. Plasmids for in vitro transcripition were prepared by use of Qiagen (Chatsworth, CA) columns. All helper plasmids (DH-BB, DH-BB-Bst, and DH-BB-ZZ) and SinRep/LacZ plasmid were linearized by XhoI restriction enzyme digestion and purified by phenol/chloroform extraction followed by ethanol precipitation. Transcription reactions were carried out by using InvitroScript Cap Kit (Invitrogen) to produce large quantities of capped mRNA transcript from the SP6 promoter. For cotransfections of helper and SinRep/LacZ RNA into BHK cells, electroporations were performed as described before[2]. Electroporated cells were transferred to 10 ml of αMEM containing 5% FCS and incubated for 12 h. Cells were then washed with PBS and incubated in 10 ml of Opti-MEM I medium (Gibco-BRL) without FCS. After 24 h, culture supernatants were harvested and aliquots were stored at -80°C.

Immunoblot assay. Cells were lysed in 20 mM Tris-HCl buffer (pH 8.0) containing 1% Triton X, 0.15 M NaCl, 1 mM phenylmethylsulfonyl fluoride, 1 mM EDTA and 10% glycerol 24 h after transfection. Cell extracts were then sonicated and mixed with electrophoresis loading buffer (125 mM Tris-HCl, pH 6.8, 10 mM β-mercaptoethanol, 2% SDS, 10% glycerol and 0.01% bromophenol blue). Virus samples were pelleted by ultracentrifugation of the supernatants (10 ml) in an SW41 Beckmann Rotor (35,000 rpm, 2 h, 4°C) and resuspended in electrophoresis loading buffer. Cell extracts and viral samples were subjected to immunoblot analysis as described before[11] by using anti-Sindbis virus mouse immune ascitic fluid (ATCC VR-1248) and horseradish peroxidase (HRP)-conjugated rabbit anti-mouse IgG antibodies (Pierce, Rockford, IL). Blot was visualized be enhanced chemiluminesence (DuPont NEN, Boston, MA).

Infection assays. Infectivity of recombinant chimeric viruses to BHK and human cell lines was determined by transfer of the Sindbis virus vector (SinRep/LacZ) that can transduce the bacterial β-galactosidase gene[16]. Viral supernatant dilutions were incubated with or without Mabs at room temperature for 1 h, then added to adherent (2×10^5) and suspension (1×10^6) cells in six-well plates. After 1 hour incubation at room temperature, cells were washed with PBS and incubated in growth medium for 24 h. Viral infection was evaluated by X-gal Staining and FACS-Gal as described below and titers were estimated in LacZ cfu per milliliter. CFU is defined in terms of cells staining blue by X-gal.

X-Gal staining and FACS-gal assay. For X-gal staining, commercial protocol was followed. Briefly, cells were fixed in PBS containing 0.5% glutaraldehyde for 15 min followed by washing with PBS three times. Then cells were stained with PBS containing 1 mg/ml X-gal, 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide and 1 mM $MgSO_4$ at 37°C for 2 h. The FACS-gal assays were performed as described[25].

Acknowledgment

This work was supported by National Institutes of Health Grants CA68498 and CA22247.

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Exhibit 7

File No.: 5986/0I124

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of:

Daniel MERUELO and Kouichi OHNO

Serial No.: 08/829,558 Group Art Unit: 1645

Filed: 3/28/97 Examiner: R. Zeman

For: VIRAL VECTORS HAVING CHIMERIC ENVELOPE PROTEINS CONTAINING THE IgG-BINDING DOMAIN OF PROTEIN A

Declaration of Daniel Meruelo Under 37 C.F.R. §1.132

DANIEL MERUELO declares:

1. I am over 21 years of age and make this declaration in support of U.S. Patent Application Serial No. 08/829,558, of which I am a co-inventor.

2. I received a Bachelor of Science degree conferred on me by Columbia University, New York, NY, in 1969 and a Ph.D. degree in Biochemistry and Immunology conferred on me by the Johns Hopkins University Baltimore, MD in 1974. I am a Professor of Pathology in the Department of Pathology at New York University Medical Center, a position which I have held since 1988. A copy of my curriculum vitae is appended herewith as Exhibit 1.

3. I have read the application and the Office Actions issued in this case and participated in a telephonic interview with the Examiner on April 25, 2001. It is my understanding that claims 1-10, 18-23 and 27 -31 of the above-identified application were rejected under 35 U.S.C. §103(a) as being obvious over Barber et al (U.S. Patent 5,591,624) and Wickham et al (U.S. Patent 5,846,782) in view of Nilsson et al (Protein Eng. 1:107-113, 1987). Based on the discussion during the interview, I understand that evidence showing the advantages of the invention, particularly ablation of natural virus targeting and retention of infectivity, particularly *in vivo*, would help to distinguish these references.

4. The experiments described below were performed by me or under my direct supervision and control. These experiments show that the viral vectors as claimed in the above-identified application target cells *in vivo* and effectively infect the targeted cells.

5. In these experiments, 10^7 human colon carcinoma cells, cell line LS1747, were inoculated subcutaneously into 8-10 week old nude mice. The LS174T cell line is a human tumor-derived cell line which is specifically a CEA (carcinoma embryonic antigen)-positive colon carcinoma cell line which can grow in BALB/c nude mice when injected as a subcutaneous inoculum.

6. After 10 days or more when tumors grew to a visible size, the animals received a single injection of a Sindbis viral vector containing the ZZ domain of Protein A inserted into the E2 protein of the Sindbis virus as disclosed in the above-identified application. (Page 25,

lines 12-14). The viral vector also contained a β-galactosidase reporter gene. (Page 21, lines 22-24). One group of animals received the viral vector preparation (250 μl of the viral vector preparation containing approximately 10^8 infectious particles) complexed with anti-CEA antibodies (20 μg). The viral vector and antibodies were complexed by incubation for 30 minutes at room temperature as disclosed on page 10, lines 33-34 of the above-identified application. Another group received the viral vector alone (250 μl of viral the vector preparation) and a third group received antibody alone (20 μg) .

7. 16-24 hours later, the tumors were harvested, teased into single cell suspensions and stained with a rhodamine-conjugated anti-CEA antibody and also exposed to fluoreacein di-β-D-galactopyranoside (FDG), a substrate for the detection of β-galactosidase in single cells by flow cytometry assay. The results are shown in Figure 1 appended herewith as Exhibit 2.

8. In Figure 1, fluorescence was measured by a Fluorescence Activated Cell Sorter (FACS) which detect signals emitted by rhodamine-conjugated anti-CEA antibody and fluoreacein di-β-D-galactopyranoside (FDG). The former signal is specific for the tumor cell and the latter signal is specific for β- galactosidase, the marker gene. Successful targeting is only indicated if both signals are present.

9. In Figure 1 tumors from mice receiving (A) only antibody and (B) only virus did not stain positively for β-galactosidase (0.11% and 0.03%, respectively). On the other hand, as shown in panel C, about 73% of tumor cells in mice injected with the virus-antibody complex were positive for β-galactosidase (upper right quadrant of Figure 1C).

10. There was no evidence that non-tumor cells were stained using the virus-antibody complex. However, it is possible that a small amount of non-specific binding occurred.

11. These data clearly show that introducing an IgG binding domain from Protein A into a viral envelope protein, in this case, Sindbis virus E2 protein, provides for highly selective targeting. Just as importantly, the targeted viruses were competent for infecting cells, since about 73% of tumor cells expressed the transgene carried by the Sindbis vector (a very high percentage for *in vivo*). Furthermore, the data show that (1) incubation of the modified viral vector with antibody yields targeted vector, and (2) vector that is not conjugated to antibody, or that loses antibody, essentially does not infect cells *in vivo*.

12. Thus, this invention improves early efforts at targeting viral vectors and achieves a level of targeting selectivity and infection efficiency that had not previously been obtained. Neither of the cited prior art references teach or even suggest these advantages, much less how to achieve them. Indeed, as discussed during the interview, the art recognized the tremendous advance achieved by the present invention (as demonstrated in the Nature Biotechnology editorial appended to the amendment filed concurrently herewith as Exhibit 5).

13. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment or both under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the above-identified application or any patent issuing thereon.

Date: 6/22/01

By: _____
Daniel Meruelo, Ph.D.

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Exhibit 1

DANIEL MERUELO, PH.D.

Home Address:	9 Country Club Lane Scarborough, New York 10510
Business Address:	New York University Medical Center Department of Pathology 550 First Avenue New York, New York 10016
Date of Birth:	March 5, 1947
Place of Birth:	Cienfuegos, Cuba (U.S. Citizen)

PROFESSIONAL EMPLOYMENT:

1988-present	Professor of Pathology
1984-1988	Tenured Associate Professor of Pathology
1982-1984	Associate Professor of Pathology
1977-1982	Assistant Professor of Pathology

EDUCATION:

9/69 - 8/74	Ph.D. degree in Biochemistry and Immunology The Johns Hopkins University Baltimore, MD.
9/64 - 6/65 2/66 - 6/69	B. S. degree Columbia University, New York, NY.

POSTDOCTORAL TRAINING:

9/74 - 8/77	Research Fellowship in Immunogenetics with Hugh O McDevitt, M.D. Stanford University, CA.

AWARDS AND HONORS:

3/89 - 2/03	NIH Merit Awardee
7/83 - 6/88	American Heart Association Established Investigator
7/78 - 6/83	Leukemia Society of America Scholar
1/78 - 12/82	Irma Hirschl Career Scientist Awardee
9/76 - 8/77	N.I.H. Postdoctoral Fellowship IF32 CA05763-01
9/74 - 8/76	Damon Runyon-Walter Winchell Postdoctoral Fellowship DRG-30-F
9/69 - 8/74	Full Time Tuition Scholarship and Fellowship, The Johns Hopkins University
9/64 - 6/69	Freshman Competitive Full-Tuition Scholarship, Columbia University and School of Engineering and Applied Sciences, renewed yearly through graduation

MAJOR COMMITTEE ASSIGNMENTS:

1997 - present	Vice President Faculty Council, New York University
1996 - present	Senator, New York University
1992	Ad Hoc NIH Study Section to Review Gene Therapy Implementation Grants.
1991-1995	Member of the Basic Sciences II Subcommittee of the Acquired Immunodeficiency Syndrome Research Review Committee
1990 - 1994	Member Academic Affairs Council, New York University
1990 - 1996	Alternate Senator, New York University
1987 - 1989	Ad Hoc Member ACS Cell and Development Committee
1986 - 1994	Chairperson Animal Care Committee
1984 - 1989	Member NIH Immunobiology Study Section
1981 - 1994	Member Scientific Advisory Panel of the N.Y.U. Institutional Grant of the American Cancer Society.
1981 - 1994	Faculty Council (N.Y.U.) Representative to Animal Care Committee.
1980 - 1990	Member Clinical Cancer Program Project Review Subcommittee.
1980 - 1984	Member N.I.H. Clinical A (CLNA) Fellowships, Fellowship Review Committee.

MEMBERSHIP IN PROFESSIONAL SOCIETIES -- PAST AND PRESENT:

American Society of Zoologists

American Association for the Advancement of Science

American Association of Immunologists

New York Academy of Sciences

American Heart Association

American Society for Microbiology

American Society of Pathologist

LECTURES

1974-1996 More than 250 invited lectures at conferences, academic institutions, and pharmaceutical/biotech companies.

FUNDING:

1977-2001 Laboratory has been continuously funded since 1977. Grants obtained from government, non-profit foundations and for-profit entities have totaled approximately $20 million in direct costs.

MAJOR RESEARCH INTERESTS:

Genetics, Immunology, Virology and Molecular Biology with specific emphasis on leukemia research.

TEACHING EXPERIENCE

Teaching Assistant, Cell Biology Laboratories, The Johns Hopkins University, 1970-1971.

Assistant Professor, New York University Medical Center, 1977-1982.

Associate Professor, New York University Medical Center, 1982-1984.

Tenured Associate Professor, New York University Medical Center, 1984-1988.

Professor of Pathology, New York University Medical Center, 1988 to present.

Teaching functions performed include (a) teaching several medical and graduate (rotations) students during summers of 1978-1986; (b) organized the graduate student seminars for 1979-1980; (c) organizing the Irvington House "Work-in-Progress" seminars; (d) ran Immunology Club Seminars 1981-82; (e) ran Pathology Seminars 1982; (f) lecturing in the graduate course in immunology and virology at intervals 1978 - 1992.

Graduate students past twenty-one years: Richard Bach, Jack Dworkin, Mary-Ann Zalman, Steven Degar, Elmer Choi, Jung Choi, Jonathan Benjamin, Maja Maric, Lisa Bastiani, and Ravi Akella.

Postdoctoral Fellows past twenty-three years: Janis Kennard, Ph.D., Christine Pampeno, Ph.D., Robert Choi, Ph.D., George Dalon Brown, Ph.D., Nuria Amari, Ph.D., Suzanne Arant, Ph.D., Toshikatsu Nobunaga, M.D.,Ph.D., Takayuki Yoshimoto, Ph.D.., Shoji Kamiura, M.D., Ph.D., Munehiro Kato, M.D., Ph.D., Yoshihiro Samejima, M.D., Kouichi Ohno, D.V.M., Hiroshi Suzuki, Ph.D, Nobuhisa Ishiguro, M.D., Yasushi Iijima, M.D., Hiroshi Ikeda, M.D., Keisuke Sawai, M.D., and Akihiro Ishizu, M.D., Ph.D.

ISSUED PATENTS

1. Lavie, D., Meruelo, D., Lavie, G., Revel, M., Vande Velde, V., and Rotman, D.: Antiviral Compositions containing aromatic polycyclic Diones and Methods for treating retroviral infections. **USA Patent** # 5,047,435, 1991.

2. Lavie, D., Meruelo, D., Lavie, G., Revel, M., Vande Velde, V., and Rotman, D.: Antiviral Compositions containing aromatic polycyclic Diones and Methods for treating retroviral infections. ATRA Patent # 0 332 679.

3. Lavie, D., Meruelo, D., Lavie, G., Revel, M., Vande Velde, V., and Rotman, D.: Antiviral Compositions containing aromatic polycyclic Diones and Methods for treating retroviral infections. BELG Patent # 0 332 679.

4. Lavie, D., Meruelo, D., Lavie, G., Revel, M., Vande Velde, V., and Rotman, D.: Antiviral Compositions containing aromatic polycyclic Diones and Methods for treating retroviral infections. CANADA Patent # 1,329,133.

5. Lavie, D., Meruelo, D., Lavie, G., Revel, M., Vande Velde, V., and Rotman, D.: Antiviral Compositions containing aromatic polycyclic Diones and Methods for treating retroviral infections. EPC Patent # 0 332 679.

6. Lavie, D., Meruelo, D., Lavie, G., Revel, M., Vande Velde, V., and Rotman, D.: Antiviral Compositions containing aromatic polycyclic Diones and Methods for treating retroviral infections. South Africa Patent # 88/5838.

7. Lavie, D., Meruelo, D., Lavie, G., Revel, M., Vande Velde, V., and Rotman, D.: Antiviral Compositions containing aromatic polycyclic Diones and Methods for treating retroviral infections. Sweden Patent # 0 332 679.

8. Meruelo, D., and Lavie, G.: Biological Fluid Purification System. **USA Patent** # 5,149,718, 1992.

9. Meruelo, D., and Lavie, G.: Biological Fluid Purification System. South Africa # SA90/0358.

10. Meruelo, D., and Lavie, G.: Biological Fluid Purification System. ASTL # 93/640125.

11. Meruelo, D., and Lavie, G.: Biological Fluid Purification System. **USA Patent** # 5,326,788, 1994

12. Meruelo, D., Lavie, G., and Mazur, Y.: Composition and Methods for treating viral infections. ASTL Patent #93/640131.

13. Meruelo, D., Lavie, G., and Mazur, Y.: Composition and Methods for treating viral infections. South Africa Patent # SA90/2032.

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of 80-90% in the platelet count within 48 h. Full recovery took 10-14 days. Virus administration induced a strong but transient erythroblastosis (peaking 24 h after administration), which settled 48 h later. Normochromic anemia occurred over the next 10 days with hemoglobin levels dropping by about 40% to reach the lowest level 10 days after administration and taking two months for full recovery."

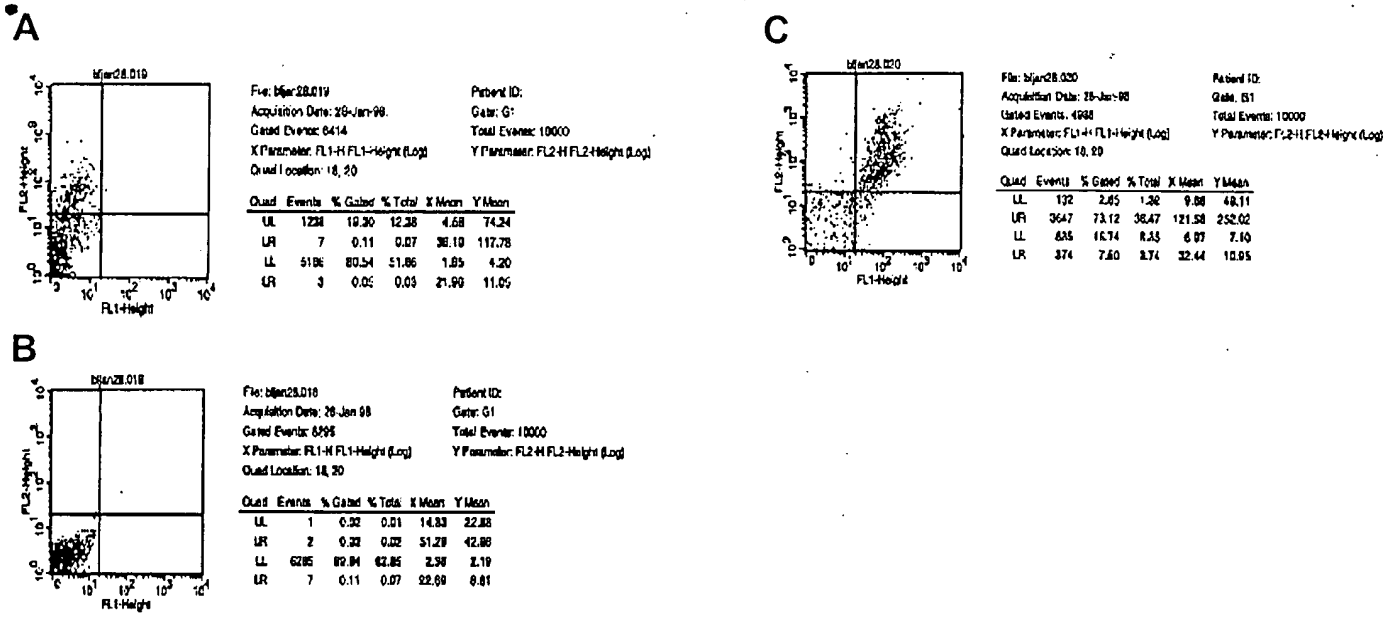

Figure 2. 10^7 human colon carcinoma cells, LS1747, were inoculated subcutaneously into 8-10 week old nude mice. When tumors grew to a visible size, animals received a single injection of Sindbis plus anti-CEA antibodies or virus alone or antibody alone. 16-24 h later the tumors were harvested, teased into single cell suspensions and stained with a rhodamine conjugated anti-CEA antibody and also exposed to fluorescein di-β-D-galactopyranoside (FDG), a substrate for the detection of β-galactosidase in single cells by flow cytometry assay. Tumors from mice receiving (A) only antibody and (B) only virus did not stain positively for β-galactosidase (0.11% and 0.03%, respectively). On the other hand, as shown on panel C, about 73% of tumor cells in mice injected with the virus-antibody complex were positive for β-galactosidase (upper right quadrant).

Finally, according to Alemany *et al.* (Alemany R, Balague C, Curiel DT. Replicative adenoviruses for cancer therapy. Nat Biotechnol. 18:723-727, 2000) although active tumor targeting of adenovirus has been achieved using antibodies or other ligands, *in vivo* stability and size of the complexes formed are serious issues greatly diminishing chances for success.

I hope this information is helpful, but do not hesitate to call me if you need anything else.

With best regards,

Daniel Meruelo, Ph.D.